Improvement of organic solvent tolerance in Escherichia coli by gene mutations

Doctor's Thesis

Improvement of organic solvent tolerance in *Escherichia coli* by gene mutations

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Preface

Our daily lives depend on petroleum products. The petroleum-based organic solvents are extensively used as raw materials and solvents for organic synthesis in the manufacture of various chemicals. Organic solvents can be toxic to organisms. Environments polluted with petroleum or synthetic organic solvents represent one definition of extreme environments for many organisms. However, some bacteria can grow in these extreme conditions containing high concentrations of highly toxic organic solvents such as toluene and xylene. Organic solvent tolerant bacteria are a relatively novel group of extremophilic microorganisms. These bacteria are being explored for their potential in industrial and environmental biotechnology. Organic solvent tolerant bacteria can be used as efficient biocatalysts in an aqueous and organic solvent two phase system. The use of two phase systems provides numerous attractive advantages in the bioconversion of organic compounds by whole-cell biocatalysts. The advantages of two-phase systems include not only the production of useful compounds from hydrophobic substrate, but also the maintenance of a low concentration of toxic or inhibitory compounds in the aqueous phase and an easier recovery of both product and biocatalyst. Although the catalytic efficiency of whole-cell biocatalysts in two-phase systems is often lowered due to the toxicity of organic solvents toward the cells, the use of organic solvent tolerant bacteria will expand the usability of these biocatalysts in an aqueous-organic solvent two phase system. The biochemical and genetic properties of the cell structures of *Escherichia coli* are well understood. This knowledge is useful

in understanding the organic solvent tolerance mechanisms. The organic solvent tolerance level of *E. coli* is relatively high among various microorganisms. In *E. coli*, the AcrAB-TolC efflux pump (bacterial nano pump) have been shown to provide intrinsic tolerance to organic solvents. This nano pump reduces the intracellular solvent concentration in *E. coli* cells exposed to organic solvents. However, the correlation between expression of this pump and organic solvent tolerance in *E. coli* was not yet fully understood.

In this dissertation entitled "Improvement of organic solvent tolerance in *Escherichia coli* by gene mutations" constitutes of four chapters and focuses on the correlation between expression of AcrAB-TolC efflux pump and organic solvent tolerance in *E. coli*. This study suggests a new strategy for increasing the organic solvent tolerance level in *E. coli* to improve the usability of the whole-cell biocatalysts in two-phase systems employing organic solvents.

Chapter 1 explains categories of organic solvent tolerant microorganisms in a wide variety of extremophiles, discovery of organic solvent tolerant bacteria, correlation between the organic solvent toxicity and its log P_{OW} value, organic solvent tolerance mechanisms of various microorganisms, and organic solvent tolerance of *E. coli*. Several solvent tolerant bacteria and their tolerant mechanisms have been reported so far. This chapter describes that knowledge. Especially, organic solvent tolerance mechanism in *E. coli* is described in detail.

Chapter 2 explains "Contributions of mutations in *acrR* and *marR* genes

to organic solvent tolerance in *Escherichia coli*". The AcrAB-TolC efflux pump plays a crucial role of maintaining inherent organic solvent tolerance in *E. coli.* Mutations in regulatory genes such as *marR*, *soxR*, and *acrR* are known to increase the expression level of the AcrAB-TolC pump. In this study, cyclohexane-tolerant *E. coli* JA300 mutants were isolated and examined by DNA sequencing for mutations in *marR*, *soxR*, and *acrR* to identify these mutations. Among 8 mutants tested, strain CH7 carried a nonsense mutation in *marR* (named *marR109*) and an insertion of IS*5* in *acrR*. This strain exhibited the highest organic solvent tolerance levels. These mutations were introduced into the *E. coli* JA300 chromosome by a site-directed mutagenesis method to clarify the involvement of these mutations in improving organic solvent tolerance. As the result, three JA300 mutants carrying *acrR*::IS*5*, *marR109*, or both were constructed. The organic solvent tolerance levels of these three mutants were increased in the following order:

JA300 < JA300 *acrR* mutant< JA300 *marR* mutant< JA300 both *acrR* and *marR mutant*. JA300 both *acrR* and *marR mutant* formed colonies on an agar plate overlaid with cyclohexane and *p*-xylene mixture. The organic solvent-tolerance level and AcrAB-TolC efflux pump-expression level in JA300 both *acrR* and *marR mutant* were similar to those in parent strain CH7. Thus, it was shown that the synergistic effects of mutations in only two regulatory genes, *acrR* and *marR*, can significantly increase organic solvent tolerance in *E. coli*.

Chapter 3 explains "Improvement of organic solvent tolerance by

disruption of the *lon* gene in *Escherichia coli*". The Lon ATP-dependent protease contributes to protein quality control and cellular homeostasis by eliminating abnormal proteins and participating in rapid turnover of several regulatory proteins. We examined the organic solvent tolerance of a ⊿*lon* mutant of *E. coli* K-12 and found that the mutant showed significantly higher organic solvent tolerance than the parent strain. ⊿*lon* mutants are known to overproduce capsular polysaccharide, resulting in the formation of mucoid colonies. We considered that this increase in capsular polysaccharide production might be involved in the organic solvent tolerance in *E. coli*. However, a ⊿*lon* ⊿*wcaJ* double-gene mutant displaying a nonmucoid phenotype was as tolerant to organic solvents as the ⊿*lon* mutant. This result suggests that capsular polysaccharide is not involved in organic solvent tolerance. Hence, the Lon protease is known to exhibit proteolytic activity against the transcriptional activators MarA and SoxS. These regulatory proteins can enhance the expression level of the AcrAB-TolC efflux pump. We found that the ⊿*lon* mutant showed a higher expression level of AcrB than the parent strain. In addition, the ⊿*lon* ⊿*acrB* double-gene mutant showed a significant decrease in organic solvent tolerance. Therefore, organic solvent tolerance in the ⊿*lon* mutant was shown to depend on the AcrAB-TolC pump but not capsular polysaccharide. *E. coli* strain JA300 *acrR*::IS *marR* overexpresses the AcrAB-TolC pump and exhibits high-level solvent tolerance. In an attempt to further improve the solvent tolerance of JA300 *acrR*::IS *marR*, a *lon* gene disruptant of this strain was constructed. However, the resulting mutant JA300 *acrR*::IS *marR* ⊿*lon*

showed lower solvent tolerance than JA300 *acrR*IS *marR*.

Finally, chapter 4 is referred to the generalization of this dissertation, and a future perspective on the study is described.

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Chapter 3

Improvement of organic solvent tolerance by disruption of the *lon* gene in *Escherichia coli*

Chapter 1

Introduction

1-1 Extremophile

Extremophiles are organisms that are adapted to grow optimally under conditions that are hostile to man. By contrast, organisms living in more moderate environments are termed mesophiles. Extremophiles are found in various environmental niches different from mesophilic conditions. These environmental niches including hot springs, cold arctic water, alkaline and acidic water, saturated salt brines, and pressurized abyssal waters, had originally been considered to be too extreme to support microbial life at all. Depending on their optimal growth conditions, some extremophiles are categorized as follows, and their correlation is pictorially summarized in Fig. 1-1. Some organisms adapted to more than two extreme conditions (Fig. 1-1).

Acidophile: an organism with optimal growth at pH levels of 3 or below
Alkaliphile: an organism with optimal growth at pH levels of 9 or above
Endolith: an organism that lives in microscopic spaces within rocks
Halophile: an organism requiring at least 1 M concentrations of salt for growth Hyperthermophile: an organism having a growth temperature optimum of more than 80°C
Thermophile: an organism that can thrive at temperatures between 60–85 °C
Hypolith: an organism that lives underneath rocks in cold deserts

Metallotolerant: an organism capable of tolerating high levels of dissolved heavy metals

Oligotroph: an organism capable of growth in nutritionally limited environments

Piezophile: an organism that lives optimally at high hydrostatic pressure

Psychrophile/Cryophile: an organism having a growth temperature optimum of 10°C or lower, and a maximum temperature of 20°C

Radioresistant: an organism resistant to high level of ionizing radiation

Xerophile: an organism that can grow in extremely dry, desiccating conditions

Organic solvent tolerant: an organism capable of growth in the presence of highly toxic organic solvents such as benzene, toluene, and xylene.

Some organic solvents, as pollutants originating from human activities, also create extreme environmental conditions, and organic solvent tolerant microorganisms are capable of tolerating such environments, and are relatively newly recognized as a subgroup of the extremophiles (43, 55, 82).

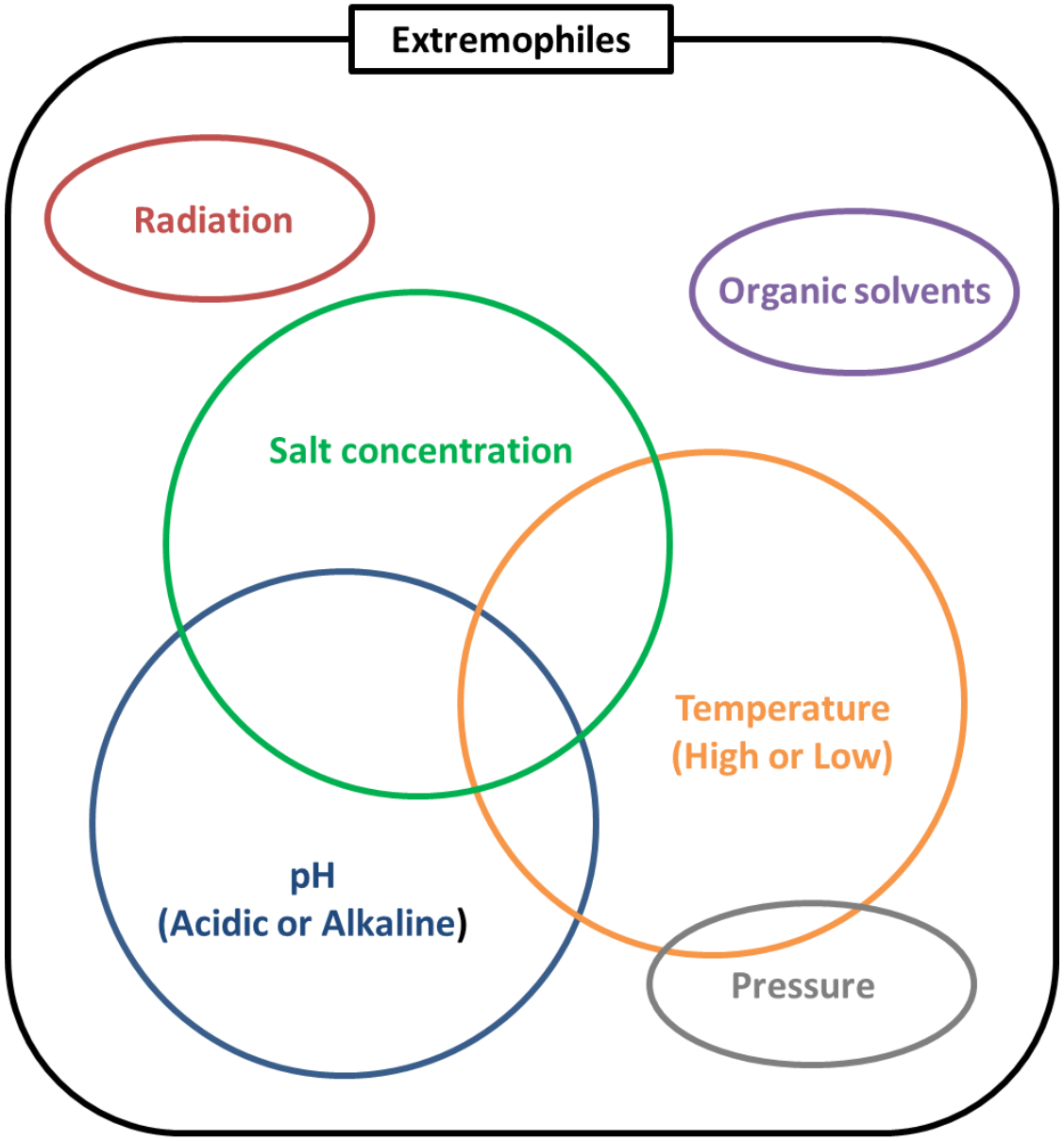


Fig. 1-1 Extremophiles
Extremophiles can grow in various extreme environments. Some of them adapted to multi extreme environments.

1-2 Discovery of organic solvent tolerant bacteria

Organic solvents are widely used to dissolve and disperse a variety of hydrophobic organic compounds such as fats, oils, waxes, and pigments. They are frequently used in the industrial manufacture of various chemicals and the laboratory processes.

Some organic solvents are known to be extremely toxic to organisms even at very low concentrations. Ecological systems are often damaged by pollution with crude oil due to an accidental marine disaster. Hydrophobic organic solvents accumulate in and disrupt the cell membrane because they can bind to the cell membrane, thereby affecting its integrity. Disruption of membrane functions implies loss of the permeability barrier and the energy transducer, and this thereby leads to growth inhibition and cell death (39, 91). Because of their toxicity, organic solvents have been used as permeabilization agents, disinfectants, food preservatives, and industrial solvents (21, 45). There are some microorganisms which can assimilate these toxic organic solvents. However, they assimilate these solvents only when the solvent concentration is very low. Any medium containing large volumes of toxic organic solvents seems to be an extreme condition for many microorganisms, and therefore for many years it was believed that no microorganism could thrive in such a harmful environment (42, 83). However, organic solvent tolerant bacteria capable of growing in the presence of these toxic solvents have been reported (42, 109).

An organic-solvent tolerant bacterium was reported by Inoue and Horikoshi for the first time. They discovered a toluene tolerant bacterium,

Pseudomonas putida IH-2000. This strain was able to grow in a two phase system containing 50% (v/v) toluene, although this bacterium was not able to use this aromatic as a carbon source (42). This report was followed by three independent studies that described the isolation of three different *P. putida* strains that tolerated related organic solvents, *e.g.* styrene (30), xylenes (20), and toluene (44). These solvent tolerant *Pseudomonas* strains opens new avenues of research into cellular metabolism (46).

Many of these solvent tolerant bacterial species were Gram-negative bacteria including *Pseudomonas* spp. (67, 71). Therefore, it has been assumed that Gram-negative bacteria are more tolerant to organic solvents than Gram-positive bacteria. However, Gram-positive bacteria such as *Bacillus* (43, 64, 91) and *Rhodococcus* (77) have also been found to exhibit organic solvent tolerance, although the mechanisms of their organic solvent tolerance are not yet fully understood. Several investigators have also been able to isolate solvent tolerant strains from solvent sensitive bacteria, suggesting that bacteria can adapt at least somewhat to the presence of organic solvents (30). A number of attempts have been made to produce valuable compounds through the bioconversion of hydrophobic organic compounds in two-phase systems consisting of an organic solvent and an aqueous medium. Organic solvent tolerant microorganisms are beneficial for process development to increase the productivity of the bioconversion in an aqueous-organic solvent two-phase system. Thus, organic solvent tolerant bacteria are being explored for their potential in industrial and environmental biotechnology (90).

1-3 Correlation between the organic solvent toxicity and its log P_{OW} value

Toxicity of organic solvents against microorganisms differs depending on the kind of organic solvents (Table 1-1). Organic solvents include a wide variety of compounds with different chemical structures, such as benzene rings and aliphatic alcohols; many of these compounds are toxic to microorganisms, plants, animals and humans. Some organic solvents hardly suppress growth of bacteria. On the other hand, benzene and toluene are highly toxic to bacteria. An evaluation method to measure the toxicity of an organic solvent in two phase systems was established by Inoue and Horikoshi for the first time. They proposed an empirical rule concerning the toxicity on the basis of colony development by various microorganisms on an agar medium. Colony development was most correlated with the P_{OW} value among several physiochemical parameters of the organic solvents. The P_{OW} value is the common logarithm of P_{OW} that is a partition coefficient of the organic solvent between *n*-octanol and water. The P_{OW} value is an index of polarity and an indicator of the toxicity of the solvent (56) (Fig. 1-2). Organic solvents with lower log P_{OW} values are more toxic to microorganisms than are solvents with relatively higher log P_{OW} values. The lowest log P_{OW} in which a strain will grow is known as the index value for that strain, and the solvent with that log P_{OW} is known as the index solvent (19). It is generally accepted that solvents with log P_{OW} values below 5 such as benzene (log P_{OW} 2.0), styrene (log P_{OW} 3.6), xylene (log P_{OW} 3.2), and toluene (log P_{OW} 2.5) are considered to be extremely toxic because of their high degree of partition into the aqueous medium layer surrounding the cells, and thus into the cell

membrane (41). The accumulation of these solvents in the cytoplasmic membrane of bacteria causes disorganization of the cell membrane structure and impairment of the vital membrane functions (Fig. 1-3) (97, 98). Organic solvents dissolved into biological membranes appear to disrupt their structure, which results in a loss of ions, metabolites, changes the intracellular pH and membrane electrical potential, and finally leads to cell death (45, 96). Nevertheless, as described above, several *Pseudomonas* species have been isolated that are able to grow on a medium in the presence of high concentrations of toxic organic solvents, such as toluene, styrene and *p*-xylene (3, 20, 42, 44, 104).

Table 1- 1 Organic solvent tolerance of several microorganisms[a]

Solvent	log P_{ow}	*Pseudomonas putida* IH-2000	*Pseudomonas putida* Px51T	*Pseudomonas putida* IFO3738	*Pseudomonas fluorescens* IFO3507	*Escherichia coli* K-12 JA300	*Bacillus subtilis* AHU-1219	*Saccharomyces uvaram* ATCC26602
n-Dodecan	7.0	+	+	+	+	+	+	+
n-Decane	6.0	+	+	+	+	+	+	−
n-Octane	4.9	+	+	+	+	+	+	−
Diphenyl ether	4.2	+	+	+	+	+	−	−
n-Hexane	3.8	+	+	+	+	+	−	−
Cyclohexane	3.4	+	+	+	+	−	−	−
p-Xylene	3.1	+	+	+	−	−	−	−
Toluene	2.6	+	+	−	−	−	−	−
Benzene	2.1	−	−	−	−	−	−	−

Results cited from Inoue and Horikoshi

[a] A cell suspention was spread on an LBGMg agar. An agar surface was overlaid with appropriate organic solvent.

+ indicated that each strain grew in the presence of the organic solvent.

− indicated that each strain didn't grow in the presence of the organic solvent.

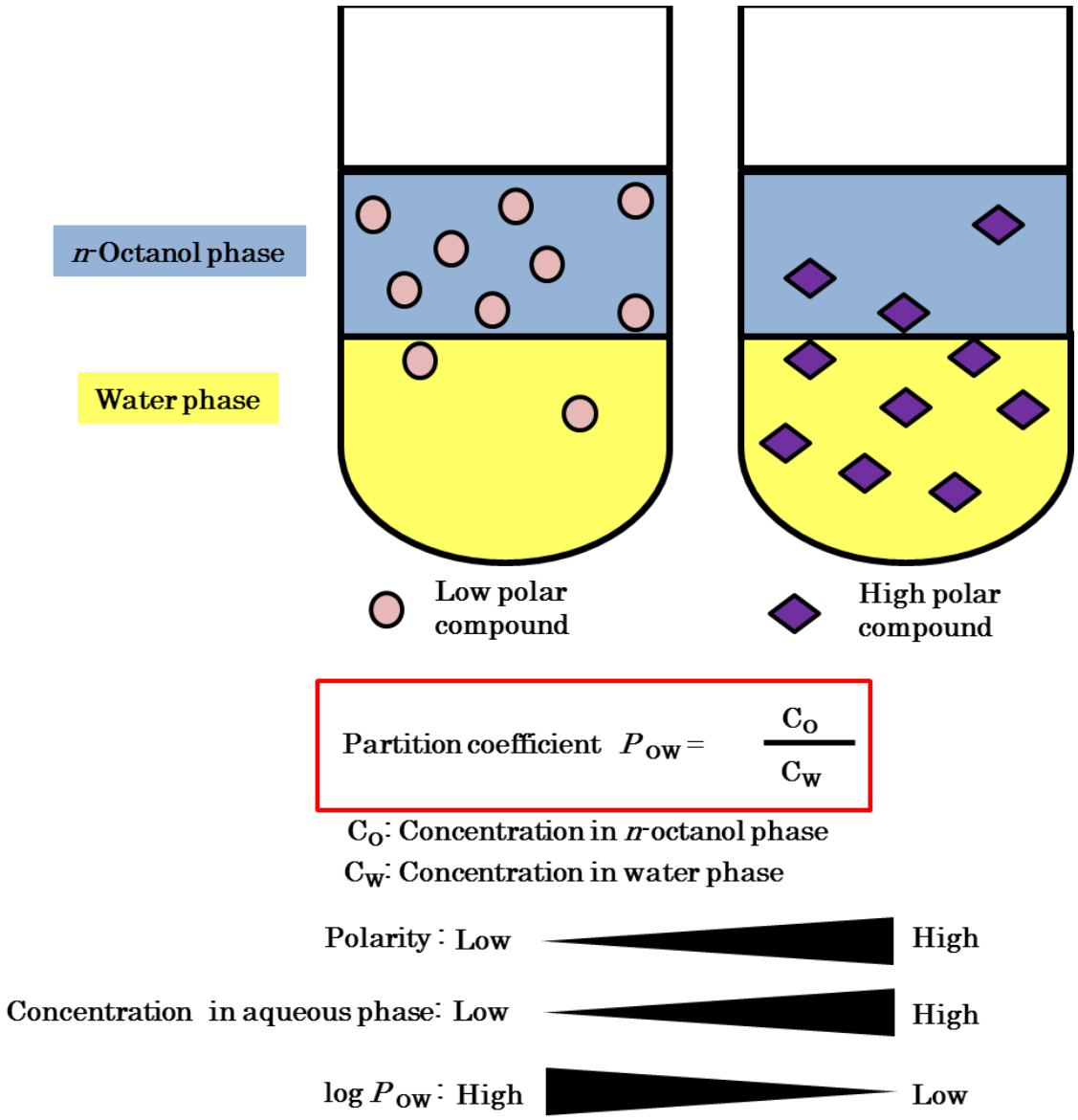
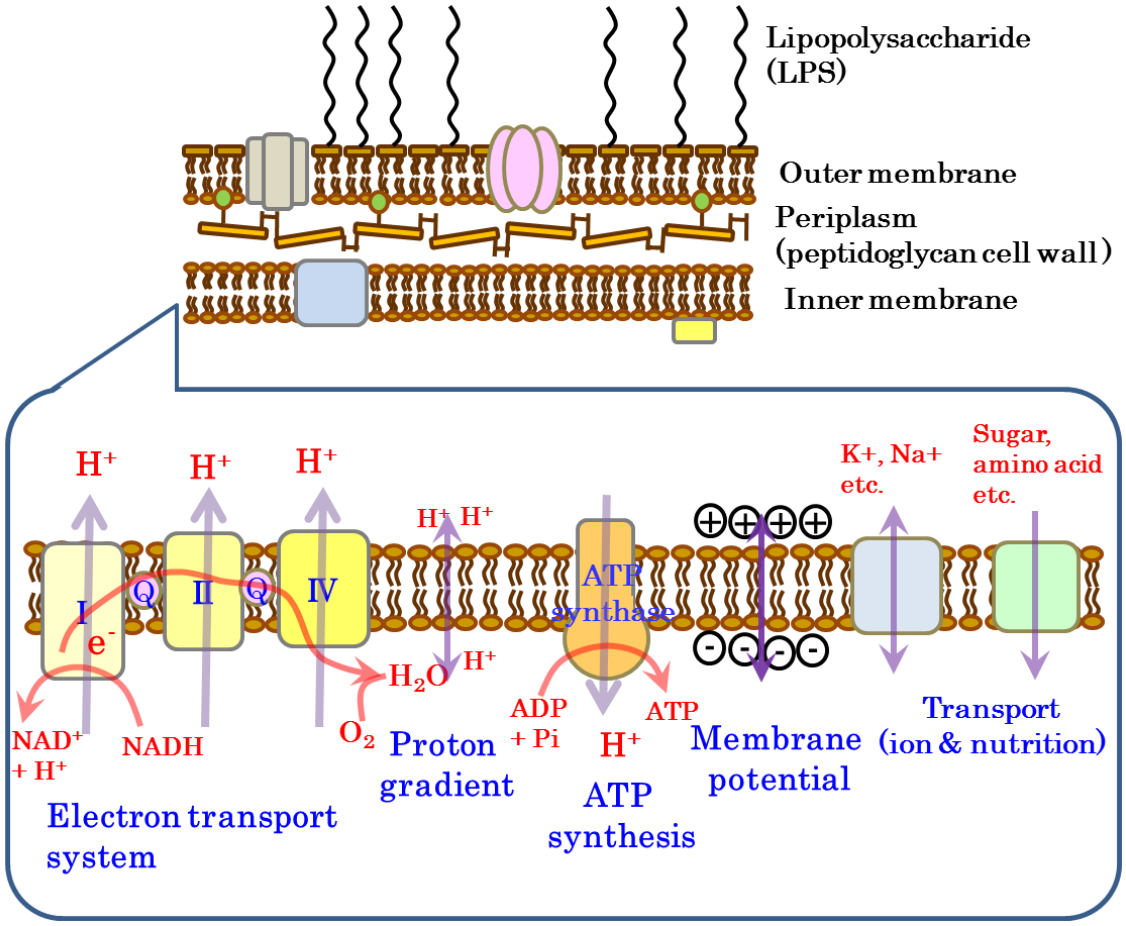


Fig. 1-2 Correlation between toxicity and polarity which is represented with $\log P_{OW}$

The P_{OW} value is the common logarithm of P_{OW} that is a partition coefficient of the organic solvent between *n*-octanol and water. The P_{OW} value is an index of polarity and an indicator of the toxicity of the solvent. Organic solvents with lower log P_{OW} values are more toxic to microorganisms than are solvents with relatively higher log P_{OW} values. Microorganisms can grow in the presence of an organic solvent whose log P_{OW} is equal to or greater than a particular value. This particular value has been referred to as the index value



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1-4 Organic solvent tolerance mechanisms

Organic solvent tolerance in bacteria is a multi-functional process that involves a wide range of genetic and physiological changes to cope with solvent damage. The mechanisms of response and tolerance toward organic solvents have been extensively studied particularly in *Pseudomonas putida* and *Escherichia coli* strains. These include changes of the energetic status, changes of the membrane's fluidity, changes in the cell wall and outer membrane, modification of surface properties, changes of metabolic flux, and active transport of solvents from the membrane into the environment by efflux systems, and modification of membrane proteins (4, 39, 43, 85, 102).

1-4-1 Changes in the cell membrane

Organic solvents intercalate and accumulate in microbial membranes, and thereby increasing membrane fluidity (14, 96). Many microorganisms modify the membrane composition by counteracting the increase in fluidity caused by the partition of the solvent into the lipid bilayer. Some bacteria belonging to the genera *Pseudomonas* and *Vibrio* respond to the solvent by accelerating isomerization of the *cis* unsaturated fatty acids to *trans* unsaturated fatty acids. This reaction is mediated by the *cis–trans* isomerize whose activity is constitutively present and is located in the periplasm (38). Bacteria acquire denser membranes by the isomerization. This allows cells to adapt immediately to several forms of environmental stress including organic solvent stress and heat stress.

In addition of changes of unsaturated fatty acids via the *cis* to *trans*

isomerization, *Pseudomonas putida* can change the saturated-to-unsaturated fatty acid ratio (2, 80) or the length of the acyl-chains (80). Increased levels of PlsX (fatty acid/ phospholipid biosynthesis) and FabF [3-oxoacyl-(acylcarrier- protein) synthase II] were found in the solvent tolerant mutant Rh8 of *Clostridium acetobutylicum*. Enhanced expression of these proteins lead to an increase in membrane saturated fatty acid content and to increased solvent tolerance (2). These fatty acid changes confer a denser membrane packing to bacteria, and thus contribute to solvent tolerance. The PlsX and FabF are involved in de novo biosynthesis of fatty acids. Therefore, this response requires long-term adaptation processes.

Changes in phospholipid head groups influence membrane's fluidity and are suggested to be also involved in solvent tolerance (85, 88, 96). In the case of *P. putida* and *E. coli*, the major classes of phospholipid are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). Changes in the head groups have been analyzed in several *Pseudomonas* species. In the case of *P. putida* S-12, the level of PE decreased and those of PG and CL increased, when this strain was grown in a chemostat culture in the presence of toluene. CL has a higher transition temperature than PE, and this leads to decrease membrane's fluidity and thus has a stabilizing effect. Similar changes in phospholipid head groups were observed in *P. putida* DOT-T1E exposed to toluene. On the other hand, The CL content in *P. putida* DOT-T1E influence the efficiency of the efflux pumps (13).

The cyclopropane fatty acids (CFAs) are known as a crucial determinant of acid and alcohol resistance in *E. coli* (17). Expression of the *cfa* synthase gene is dependent on the RpoS sigma factor and it takes place when cells enter the stationary phase (17, 78). It has been reported that the CFAs are involved in solvent tolerance of *Pseudomonas* species. A *cfaB* mutant of *P. putida* DOT-T1E was more sensitive to toluene shock than the parental strain (79). In contrast with other genes involved in the toluene tolerance, the *cfaB* expression is not enhanced in response to toluene in *P. putida* (27). In addition, a *C. acetobutylicum* strain overexpressing the *cfa* synthase gene showed increased butanol tolerance (110). CFA synthase levels in *C. acetobutylicum* increased after the addition of butanol (2).

Comparative membrane proteome analysis of the *C. acetobutylicum* suggested that stabilization of the membrane structure and surface is a requirement of increased solvent tolerance (61). Goodarzi et al. (35) also pointed out the importance of a number of cell-wall structural components in solvent tolerance. *E. coli* mutants in *slt* which encodes a soluble lytic murein transglycosylase or strains overexpressing *murB* which encodes a UDP-*N*-acetylenolpyruvoylglucosamine reductase, showed higher survival rates at 7% ethanol than the wild-type.

Flagellar biosynthesis is also involved in organic solvent tolerance in several bacteria. Two proteins FlgE and Hag involved in flagellar assembly and synthesis were downregulated in the butanol-tolerant mutant from *C. acetobutylicum* Rh8 (61). In addition, proteins related to flagellar biosynthesis were downregulated in *E. coli* cells exposed to ethanol and in *P.*

putida KT2440 in the presence of toluene (22, 40). By contrast, the expression level of flagellar genes was kept at a high level in *P. putida* DOT-T1E (57). Involvement of flagellar proteins in tolerance has been previously described in other solvent-tolerant *P. putida* strains (48, 92). The knockout- mutants in specific flagellar genes in these strains showed diminished solvent tolerance.

Some *P. putida* strains produced membrane vesicles composed of phospholipids, exopolysaccharides and proteins, when they are exposed to toxic chemicals. This has been proposed to serve as an active mechanism to release solvents accumulated in the cell surfaces (52), or to modify the hydrophobicity of cell surfaces. *P. putida* DOT-T1E cells increased their hydrophobicity, shifted their outer membrane lipopolysaccharide composition toward more hydrophobic and lower molecular forms and released vesicles in response to long chain alkanols (12).

1-4-2 Heat stress response

Most of the proteomic and transcriptomic assays in bacteria indicated that proteins related to heat stress response (*groES*, *groL*, *grpE*, *dnaK*) were overexpressed in the presence of solvents such as ethanol, butanol, toluene, or xylene (2, 22, 35, 40, 87, 93, 103, 108). The *rpoH* regulon is upregulated in the presence of several alcohols (16, 89). Organic solvents disturb protein folding in the cytoplasm and periplasm, thus it is assumed that the production of various chaperones is necessary to repair denatured proteins.

1-4-3 Oxidative stress response

Several studies have also shown that alcohols and aromatic compounds generate oxidative agents, and this leads to activate the response against the oxidative stress. In the presence of organic solvents, the electron transport systems do not operate adequately, and this causes an increase in the production of hydrogen peroxide and other reactive oxygen species (16, 22, 40). In response to oxidative stress, several genes belonging to the OxyR regulon are induced by toluene in *P. putida* KT2440 (22), and genes regulated by OxyR or NrdR are upregulated also in *E. coli* ethanol tolerant strains (40).

1-4-4 Efflux pumps

Efflux pumps belonging to the RND family are considered to be the most efficient mechanism of solvent tolerance in Gram-negative bacteria(85). RND transporters are proton-driven efflux systems constituted by three proteins which form a multicomponent complex extending from the inner membrane to the outer membrane (29, 70). Three kinds of RND transporters are identified in *P. putida* DOT-T1E. The genes encoding for these transporters are named *ttgABC* (toluene tolerance genes), *ttgDEF*, and *ttgGHI*. The TtgABC and the TtgGHI pumps were involved in the efflux of toluene, styrene, xylene, ethylbenzene, and propylbenzene. On the other hand, TtgDEF efflux pump was involved in the efflux of styrene and toluene only. The deduced amino acid sequences of these proteins are similar to the AcrAB-TolC efflux pump in *E. coli* and the MexAB-OprM pump of *P. aeruginosa*. The AcrAB-TolC efflux pump in *E. coli* is discussed below.

1-4-5 Organic solvent tolerance in *Escherichia coli*

E. coli is a Gram-negative, facultative anaerobic, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. *E. coli* can be grown easily in a laboratory setting, and has been intensively investigated so far. *E. coli* is the most widely studied prokaryotic model organism, and an important species in the fields of biotechnology and microbiology. The adaptation and tolerance mechanisms toward organic solvents, particularly in *Pseudomonas putida* and *Escherichia coli* strains, have been extensively studied as described previous section. Owing to the wealth of biochemical, metabolic and genetic knowledge associated with *E. coli*, *E. coli* strains are useful as a model bacterium in understanding the organic solvent tolerance mechanisms. Although *E. coli* is less tolerant to organic solvents than several *Pseudomonas* spp., its tolerance level is relatively high among various microorganisms (4). *E. coli* strains generally are able to form colonies on an agar medium overlaid with *n*-hexane (log P_{OW} 3.9) but not on the medium overlaid with organic solvents with log P_{OW} below 3.9. Several organic solvent tolerant mutants were isolated from various *coli* strains (4). These organic solvent tolerant mutants displayed improved tolerance to organic solvents such as cyclohexane (log P_{OW} 3.4) and *p*-xylene (log P_{OW} 3.1). Analysis of the mechanisms of these organic solvent tolerant *E. coli* mutants revealed that *E. coli* has genes involved in determining organic solvent tolerance levels. Various mechanisms implicated in the solvent-tolerance of *E. coli* have been reported so far. These mechanisms include energy-dependent efflux systems (19, 53, 102),

lipopolysaccharides (82), a composition of fatty acids (82), maintenance of the proton motive force (51), and an alkyl hydroperoxide reductase (32). In addition, transcriptional analysis using DNA microarrays revealed that genes such as *glpC*, *purR manXYZ*, and *crp* coding for genes related to the metabolic pathway of carbon catabolism, are implicated in the organic solvent tolerance in *E. coli* (74, 75, 94, 95). Furthermore, heterologous expressions of chaperones from *Pyrococcus horikoshii* and *Bacillus psychrosaccharolyticus* were found to improve the tolerance of *E. coli* to organic solvents (47, 73). The genes involved in organic solvent tolerance in *E. coli* are listed in Table 1-2, and solvent tolerance mechanisms are summarized below.

Table 1-2 Genes involved in organic solvent-tolerance in *Escherichia coli*

Category	Gene	Involvement of the gene in the solvent-tolerance in *E. coli*	Ref.
Efflux pumps	*acrAB*	*acrA* and *acrB* code for AcrA, a periplasmic lipoprotein and AcrB, a proton-substrate antiporter protein, respectively. These proteins are components of the AcrAB-TolC multidrug efflux pump. The AcrAB-TolC efflux pump belonging to the resistance/nodulation/cell division (RND) family have been shown to provide intrinsic tolerance to organic solvents.	(117)
	tolC	*tolC* codes for an outer membrane protein which is a component of the AcrAB-TolC efflux pump.	(11)
	acrEF	*acrE* and *acrF* code for AcrE, a membrane fusion protein and AcrF, a transporter protein. AcrE and AcrF are highly homologous to AcrA and AcrB, respectively.	(61)
	yhiUV	*yhiU* and *yhiV* code for YhiU, a putative membrane protein and YhiV, a putative transporter. YhiU and YhiV exhibites significant homology to AcrA and AcrB, respectively.	(117)
	emrAB	*emrAB* code for EmrA, a membrane fusion protein, and EmrB, a major facilitator superfamily (MFS) transporter. The EmrAB-TolC system is responsible for the efflux of various toxins. The EmrAB-TolC pump seemed to be involved in nonane- and octane-tolerance in the DacrAB mutant.	(117)
Regulators for theexpression of efflux pump	*marA*	*marA* codes for a DNA-binding transcriptional activator MarA which is a transcriptional activator that controls the expression of *marA/soxS/rob* regulon genes. *acrAB* and *tolC* are *marA/soxS/rob* regulon genes. Thus, the overexpression of MarA enhances the expression level of the AcrAB-TolC efflux pump.	(14)
	marR	MarA is transcriptionally regulated. Transcription of the *marRAB* is repressed by MarR, whereas it is autoactivated by MarA. Mutations in *marR* enhance the expression level of the AcrAB-TolC efflux pump and thus result in the increase of organic solvent-tolerance level.	(14)
	robA	*robA* codes for a DNA-binding transcriptional activator belonging to the AraC subfamily. The overexpression of Rob protein enhances the expression level of the AcrAB-TolC efflux pump.	(76)
	soxS	*soxS* codes for a DNA-binding transcriptional activator belonging to the AraC subfamily. SoxS is implicated in the regulation of superoxide response regulon. The overexpression of SoxS protein enhances the expression level of the AcrAB-TolC efflux pump.	(77)
	soxR	SoxS is transcriptionally regulated. Transcription of the *soxS* is repressed by SoxR. Mutations in soxR enhance the expression level of the AcrAB-TolC efflux pump, and this lead to the increase of organic solvent-tolerance level.	(59)

Category	Gene	Involvement of the gene in the solvent-tolerance in *E. coli*	Ref.
Maintenance of the proton motive force	*pspA*	*pspA* codes for a phage-shock protein which is induced under extreme stress conditions. This protein is involved in *maintenance* of the protonmotive force. The survival frequency of *E. coli* exposed suddenly to *n*-hexane was improved by introduction of a plasmid containing the *psp* operon.	(59)
LPS	*ostA*	*ostA*/*imp* codes for a protein involved in the transport of lipopolysaccharides to the cell surface.	(7, 82)
Carbon catabolism	*glpC*	*glpC* codes for a subunit of GlpABC, an anaerobic glycerol-3-phosphate dehydrogenase. An *E. coli* strain carrying a plasmid containing *glpC* enhanced the colony-forming frequency on an agar medium overlaid with *n*-hexane.	(108)
	fruA	*fruA* codes for a fructose-specific transport protein. An *E. coli* strain carrying a plasmid containing *fruA* increased the colony-forming frequency on an agar medium overlaid with *n*-hexane.	(108)
	purR	*purR* codes for a DNA-binding transcriptional repressor. Most genes of the *purR* regulon are involved in the nucleotide metabolism. An *E. coli* strain carrying a plasmid containing *purA* exhibited the increased organic solvent-tolerance on an agar medium overlaid with *n*-hexane.	(107)
	manXYZ	*manXYZ* operon codes a sugar transporter of the phosphotransferase system. Overexpression of the *manXYZ* can enhance the organic solvent-tolerance in the presence of *n*-hexane.	(86)
	crp	*crp* codes for a cyclic AMP receptor that acts as a DNA-binding transcriptional regulator. Disruption of the *crp* gene resulted in the increase of organic solvent-tolerance on an agar medium overlaid with the solvent mixture of *n*-hexane and cyclohexane (1:1).	(85)
	cyaA	*cyaA* codes for an adenylate cyclase involved in cAMP biosynthetic processes. Deletion of the *cyaA* gene leaded to increase of organic solvent-tolerance on an agar medium overlaid with the solvent mixture of *n*-hexane and cyclohexane (1:1).	(85)
	gadB	*gadB* codes for a glutamate decarboxylase B subunit. Disruption of *gadB* decreases the solvent-tolerance in *E. coli* strain.	(85)
	nuoG	*nuoG* codes for a *peripheral* subunit of NADH:ubiquinone oxidoreductase. Disruption of *nuoG* decreases the solvent-tolerance of *E. coli* strain.	(85)
Alkyl hydroperoxide reductase	*ahpCF*	*ahpCF* codes for an alkyl hydroperoxide reductase. A tetralin-tolerant *E. coli* containing a mutation in *ahpCF* was tolerant to cyclohexane, propylbenzene, and 1,2-dihydronaphthalene.	(35)

1-4-5-1 Energy-dependent efflux systems

A number of bacteria bear *multidrug efflux systems* to withstand various environments containing toxic compounds such as antibiotics and endogenous metabolic products. Multidrug efflux pumps are membrane proteins that extrude a variety of organic compounds actively out of the cell. Most bacteria possess several kinds of multidrug efflux pumps. Multidrug efflux pumps have been characterized by sequence homology. These include the ATP binding cassette (ABC) family, multidrug and toxic compound exporters (MATE), the small multidrug resistance (SMR) family (a member of the much larger drug/metabolite transporter superfamily), resistance-nodulation-division proteins (RND), and the major facilitator superfamily (MFS). Energy-dependent efflux systems belonging to the RND family play an important role in the maintenance solvent tolerance in gram-negative bacteria (19, 49, 59, 84, 102). The AcrAB-TolC efflux pump, a member of the RND family, is a major pump exporting various hydrophobic compounds in *E. coli* (33, 37). AcrA, AcrB and TolC are a membrane fusion protein anchored to the cytoplasmic membrane, a transporter protein located in the cytoplasmic membrane acting as an energy-dependent extrusion pump, and an outer membrane protein that is supposedly a channel which circumvents the outer membrane, respectively. Spontaneous cyclohexane-tolerant mutants from JA300 enhanced expression of AcrA and TolC (9, 102). However, the deletion of *acrAB* and *tolC* decreases the solvent tolerance of *E. coli*. The amount of solvent entering *E. coli* cells was measured after the cells were incubated in an organic solvent-aqueous two

phase system (102). In wild type strain JA300, the intracellular levels of solvents with a log P_{OW} higher than 4.4 were maintained at low levels. In contrast, the ⊿*tolC* or ⊿*acrAB* mutants accumulated the solvents more abundantly than the parent stain.

E. coli possesses several efflux pumps belonging to the RND family other than the AcrAB-TolC efflux pump. AcrE and AcrF are highly homologous to AcrA and AcrB, respectively. The *acrEF* genes are expressed under laboratory conditions at low levels. *E. coli* strain OST5500 is hypersensitive to solvents because *acrB* was defective by the insertion of IS30 (53). Suppressor mutants that were isolated from OST5500 showed high-level organic solvent tolerance. These mutants produced high levels of AcrE and AcrF proteins, which were not produced in OST5500, and in each mutant an insertion sequence (IS1 or IS2) was found integrated upstream of the *acrEF* operon. The suppressor mutants lost solvent tolerance by inactivation of the *acrEF* operon. The solvent hypersensitivity of OST5500 was suppressed by introduction of the *acrEF* operon with IS1 or IS2 integrated upstream of the operon but not by introduction of the operon lacking the integrated IS. These results indicated that IS integration activated *acrEF*, resulting in functional complementation of the *acrB* mutation.

YhiU and YhiV are also homologous to AcrA and AcrB, respectively. There proteins are thought to be components of a multidrug transporter. JA300⊿*acrAB* cells improved colony forming efficiency in the presence of nonane by transformation of a plasmid containing *yhiUV* (102). However, this recombinant was sensitive to octane and *n*-hexane. Since JA300⊿*acrAB*

cells harboring a plasmid containing *acrAB* or *acrEF* acquired tolerance against octane and *n*-hexane, the expression of *yhiUV* seemed not to be as effective in improving the organic solvent tolerance levels as the expression of *acrAB* or *acrEF*.

The Emr transporter system is known to extrude various drugs. This system is comprised of EmrB (a MFS transporter), EmrA (a membrane fusion protein) and TolC (an outer membrane channel). Although *E. coli* JA300⊿*acrAB* was sensitive to nonane and octane, JA300⊿*acrAB* harboring a plasmid containing *emrAB* improved the nonane or octane-tolerance levels (102). The Emr transporter seemed to confer tolerance on *E. coli* to weakly toxic solvents such as nonane and octane. However, *emrAB* disruptants derived from JA300 and JA300⊿*acrAB* were as tolerant to solvents as JA300 and JA300⊿*acrAB*, respectively.

1-4-5-2 Regulators for the expression of AcrAB-TolC efflux pump

Aono et al. cloned three genes, *marA, robA* and *soxS,* elevating the organic solvent tolerance of *E. coil* strain JA300 by the shotgun method from the JA300 chromosome itself (10, 66, 68). These genes code for DNA-binding proteins that are transcriptional activators belonging to the AraC subfamily with the helix-turn-helix motif. The products of these genes are known to be positively regulating the expression of several genes belonging to the *mar-sox* regulon. *marA* and *soxS* confer tolerance on *E. coli* to multiple antibiotics and superoxide anion. It was shown that the expression of the AcrAB-TolC system was positively regulated by high levels of the expression

of *marA, robA* and *soxS* (5) (Fig. 1-4). Transcription of the *marRAB* is repressed by MarR, whereas it is autoactivated by MarA. *soxS* transcription is repressed by SoxR and enhanced by the activated form of SoxR after exposure to superoxides or nitric oxide (81). Mutations in *marR* or *soxR* were suggested to enhance the expression level of the AcrAB-TolC efflux pump (9, 54, 76, 86). Cyclohexane-tolerant mutants were defective in *marR*, a repressor protein for *mar* operon including *marA* (10). In addition, *acrAB* expression is modulated locally by the repressor AcrR (60). Thus, mutations in *acrR* can lead to the enhanced expression of AcrAB.

1-4-5-3 Maintenance of the proton motive force

A phage-shock protein, PspA, was strongly induced in *E. coli* strain grown in a liquid medium overlaid with *n*-hexane or cyclooctane (51). PspA has been reported to be induced in *E. coli* cells under extreme stress conditions, and is involved in maintenance of the proton motive force under stress conditions (50). *E. coli* strain overexpressing the *psp* operon improved the survival frequency of cells exposed suddenly to *n*-hexane, but not the growth rate of cells growing in the presence of *n*-hexane.

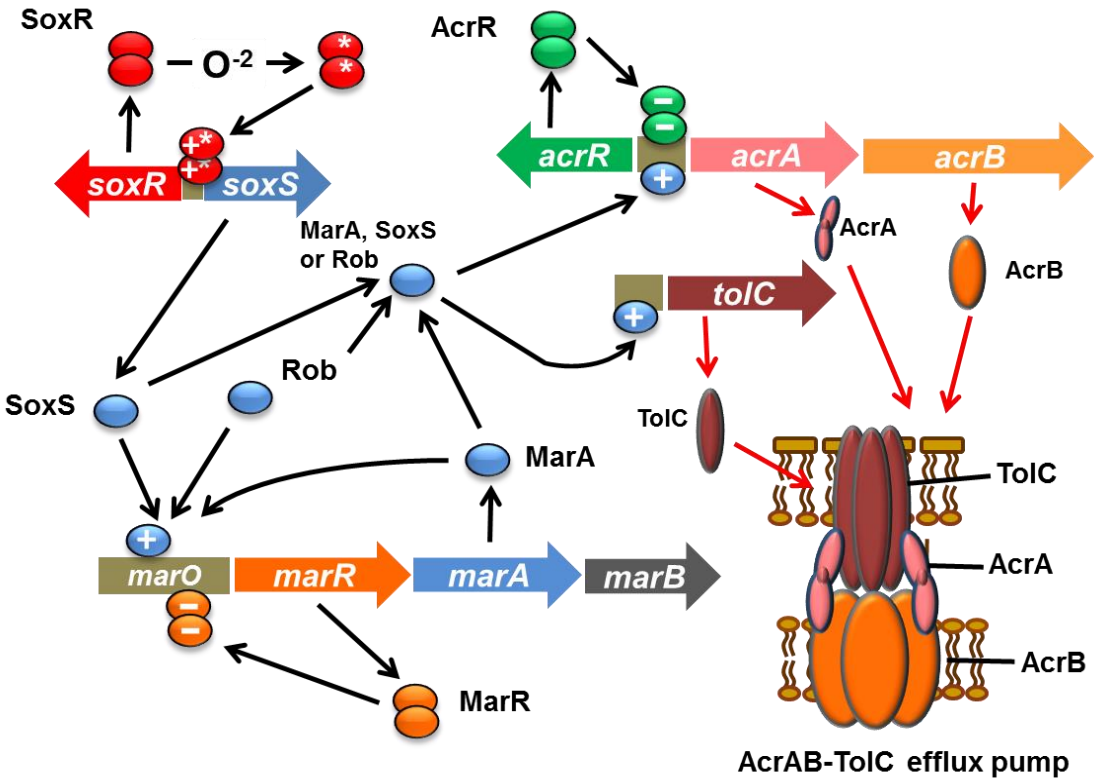

Fig. 1-4 Regulatory controls governing the expression of the *E. coli acrAB* and *tolC* genes

The Production of AcrA and AcrB is repressed by the local dimeric repressor protein AcrR. Activation of *acrAB* and *tolC* transcription occurs because of the global regulatory proteins MarA, SoxS, and Rob, any one of which can bind to a marbox upstream of these genes. The intracellular level of MarA is controlled by MarR, a dimeric protein which binds to *marO* and represses the expression of its own gene and the two others that constitute the *marRAB* operon. MarA protein can bind as a monomer to the *marO* marbox upstream of the *marRAB* promoter, where it activates transcription of *marRAB* and enhances the production of MarA. The highly elevated intracellular levels of MarA can then bind to marboxes adjacent to the promoters of *mar* regulon genes, such as *acrAB* and *tolC*, and activate their transcription. The MarA homologs SoxS and Rob can also bind to the *marO* marbox and activate *marRAB* transcription. SoxS is only produced upon conversion of the SoxR effector protein into its active form (SoxR*) by superoxide-generating agents. Rob can also activate the expression of some genes that belong to the *mar* regulon, such as *acrAB*.

1-4-5-4 Lipopolysaccharides

Genetic analysis of the *n*-hexane-tolerant strain JA300 and its *n*-hexane-sensitive derivative OST4251 showed that the *ostA* (organic solvent tolerance) is implicated in the maintenance of organic solvent tolerance in strain JA300 (8). The *ostA* was shown to be identical to the *imp* (increased membrane permeability) gene (7, 72). There were no mutations in the nucleotide sequence of the *ostA* structural gene of OST4251 compared to that of JA300. Instead, IS2 was integrated in upstream of *ostA* gene in OST4251. The insertion of IS2 at this position leaded to disrupt the putative promoter sequence. Western blotting analysis indicated that the expression level of OstA protein was significantly decreased in OST4251. These results showed that the *n*-hexane-sensitivity of OST4251 was caused by the lower production of the OstA production.

OstA is a minor protein associated with the outer membrane known to be essential for growth in *E. coli*. OstA mediates the transport of lipopolysaccharides (LPSs) to the cell surface (15). These results indicated that cell envelope biogenesis might be involved in the organic solvent tolerance in *E. coli*.

Analysis of cell surface properties in organic solvent tolerant *E. coli* mutants indicated that the cell surface of each solvent-tolerant *E. coli* mutant was less hydrophobic than that of the parent strain. LPS contents were higher in the solvent-tolerant mutants than in the parent strain. Therefore, this reduced hydrophobic property of cell surface in the mutants seemed to be caused by the increase of the content of LPS. Organic solvents

bind readily to *E. coli* cells in response to the property of the solvent. The solvent-tolerant mutants were bound less abundantly with the organic solvent than was the parent.

1-4-5-5 Carbon catabolism

Transcriptional analysis using DNA microarrays revealed that genes related to the metabolic pathway of carbon catabolism are implicated in the organic solvent tolerance in *E. coli* (74, 75, 94, 95). Gene expression profiles were collected from several organic solvent tolerant mutants before and after exposure to organic solvents. Among several genes showing higher gene expression, the overexpression of the *glpC* gene improved the colony forming efficiency of *E. coli* strain JA300 in the presence of *n*-hexane. In addition, the overexpression of *glpC* in the *E. coli* strain decreased the hydrophobicity of the cell surface. The gene *glpC* is one of the genes of the *glpABC* operon encoding the anaerobic glycerol-3-phosphate dehydrogenase (18). The GlpC subunit functions as the membrane anchor for the catalytic GlpAB dimmer.

The gene *fruA* was also upregulated in the solvent-tolerant strains after exposure to organic solvents. Overexpression of *fruA* slightly enhanced the colony formation efficiency of the *E. coli* strain in the presence of *n*-hexane. Since FruA, as well as GlpC, is a membrane-associated protein (34), it is likely that the expression of *fruA* increases the organic solvent tolerance through a change in the cell surface properties.

Genes of *purR* regulon were strongly repressed after exposure to organic solvent (94).

The *purR* gene codes for a purine nucleotide synthesis repressor. Most genes of the *purR* regulon function as the enzymes of nucleotide metabolism. Overexpression of *purR* in strain JA300 increased the colony forming frequency in the presence of *n*-hexane. In contrast, deletion of *purR* decreases the solvent-tolerance of *E. coli*.

The expression levels of the *manX*, *manY*, and *manZ* genes in *E. coli* were strongly upregulated after exposure to organic solvents (75). *E. coli* strain JA300 overexpressing *manXYZ* significantly increased colony forming efficiency in the presence of *n*-hexane. The *manXYZ* operon codes a sugar transporter of the phosphotransferase system (75). *E. coli* strain JA300 overexpressing *manXYZ* significantly increased colony forming efficiency in the presence of *n*-hexane. The *manXYZ* operon codes a sugar transporter of the phosphotransferase system (28). The quantification of the intracellular *n*-hexane showed that the level of intracellular *n*-hexane was kept lower in *E. coli* cells overexpressing *manXYZ* after incubation with *n*-hexane. It was assumed that overexpression of *manXYZ* prevented the entry of the organic solvent into the cells and thus enhanced organic solvent tolerance. Adherence of the cells expressing *manXYZ* to hydrocarbons was also investigated in a two-phase mixture consisting of organic solvents and aqueous buffer. The result showed that *E. coli* cells expressing *manXYZ* can bind more abundantly to various organic solvents compared to the control cells. Expression analysis of ManXYZ showed that these proteins are localized in cell membrane. These results indicated that the expression of *manXYZ* changed the cell surface property to obtain a higher affinity to

hydrocarbons.

Since the *glpC, purR* and *manXYZ* genes were implicated in the metabolic pathway of glucose catabolism, changes in metabolic flux was assumed to affect the organic solvent tolerance of *E. coli*. In *E. coli*, seven global transcriptional regulators (*i.e., arcA, arcB, cra, crp, cyaA, fnr,* and *mlc*) are involved in carbon catabolism (62). The organic solvent tolerances in the knockout mutants of these seven global regulator genes were investigated to identify the metabolic pathway related to organic solvent tolerance (74). Among these genes, the disruption of *crp* and *cyaA* enhanced organic solvent tolerance in *E. coli*. The *crp* and *cyaA* genes code for a cyclic AMP receptor protein and an adenylate cyclase, respectively. Crp and cAMP, synthesized from Cya, are involved in catabolite repression in *E. coli*. Therefore, it was speculated that the formation of the cAMP-Crp complex is related to the solvent-tolerance. *Δcrp* and *ΔcyaA* mutants exhibited higher expression levels of *gadB* and *nuoG* than the wild type strain. *gadB* and *nuoG* code for a glutamate decarboxylase B subunit (99) and a peripheral subunit of NADH:ubiquinone oxidoreductase (31), respectively. *ΔgadB* and *ΔnuoG* mutants decreased the organic solvent tolerance levels. This result suggested that the expressions of these proteins are involved in the organic solvent tolerance in the *Δcrp* and *ΔcyaA* mutants. GadB and NuoG function as proton transporters. Therefore, proton transport might affect the organic solvent tolerance in *E. coli*.

1-4-5-6 Alkylhydroperoxide reductase

Tetralin (1,2,3,4-tetrahydronaphthalene)-tolerant *E. coli* mutant exhibited tolerance also to cyclohexane, propylbenzene, and 1,2-dihydronaphthalene (32). The wild type strain was not tolerant to these solvents. The gene involved in the solvent-tolerance was cloned from a recombinant library from mutant DNA and identified as alkylhydroperoxide reductase operon *ahpCF*. A mutation was localized to a substitution of valine for glycine at position 142 in the coding region of *ahpC*, which is the gene that encodes the catalytic subunit of the gene. The *ahpC* mutant was found to have three times higher activity compared with that of the wild type strain in reducing tetralin hydroperoxide to 1,2,3,4-tetrahydro-1-naphthol. These results showed that the toxicity of solvents such as tetralin seemed to be caused by the formation of toxic hydroperoxides in the cell. The *ahpC* mutation increased the enzyme activity and acquired the tolerance to hydrophobic hydroperoxides.

1-4-5-7 Other mechanisms of organic solvent tolerance in *E. coli*

Supplements of alkaline earth ions such as Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} enhance the organic solvent tolerance levels in *E. coli*. These ions exhibited the maximum effects at 5 to 10 mM when *E. coli* strain was grown in LBG medium (1% Bacto tryptone, 0.5% yeast extract, 1% NaCl, 0.1% glucose). It was assumed that these divalent cations might stabilize the membrane structure by reduction of charge repulsion between anionic molecules such as LPS in membranes.

The expression of two genes, *ostB* and *ostC*, improved the organic solvent

tolerance level of the *ostA*-defective *E. coli* mutant (4). By contrast, these genes did not improve other strains. *ostB* encodes a 26-kDa cytoplasmic protein having a helix-turn-helix motif like a GntR family protein. OstB seemed to regulate the expression of *ostC*. OstC is thought to encode a 52-kDa inner membrane protein having 14 transmembrane domains. The OstC protein is homologous to AraE family proteins that might be a H^+/drug antiporter. This gene seems to correspond to the *bglT* gene.

Hydrophobic ß-lactam antibiotics passed through OmpF channels faster than through OmpC channels (69). Thus, the OmpF protein levels impact on the susceptibility of *E. coli* to several antibiotics. The levels of OmpF protein were markedly decreased in the *E. coli* mutants because of mutations in *marR* (11). OprF porin protein was reported to be absent in a toluene-tolerant mutant of *Pseudomonas aeruginosa* (58). It thus seemed likely that organic solvent molecules could also pass through the OmpF porin. Therefore, it was considered likely that the decreased levels of OmpF or loss of OmpF might contribute to improving the organic solvent tolerance of *E. coli*. The organic solvent tolerance of *E. coli* was measured under conditions in which the OmpF levels were controlled by various means as follows: alteration of NaCl concentration in the medium, transformation with a stress-responsive gene (*marA*, *robA*, or *soxS*), or disruption of the *ompF* gene (11). These results indicated that the solvent tolerance of *E. coli* did not depend on the OmpF levels in the membrane.

1-5 Applications of organic solvent tolerant bacteria in an aqueous-organic solvent two-phase bioconversion system

Whole-cell biocatalysts are beneficial in the bioconversions involving in their internal cofactor regeneration and requiring multi-step metabolic pathways. Bioconversions of hydrophobic compounds using whole-cell biocatalysts have been studied in aqueous-organic solvent two-phase systems (Fig. 1-5). There are a number of the advantages of the two-phase bioconversion systems as follows:

(1) Increase of the hydrophobic substrate concentration

(2) Maintenance of a low concentration of toxic or inhibitory compounds in the aqueous phase which reduces substrate and/or end-product inhibition

(3) Easier recovery of both product and biocatalyst

(4) Prevention of product and/or substrate hydrolysis

(5) Reduction of mass transfer limitations

(6) Alteration of substrate/product partitioning

(7) Reduction of the culture scale

(8) Prevention of the bacterial contamination

However, there are also disadvantages in the two-phase system as follows:

(1) Inhibitory effect on the biocatalyst

(2) Low reaction rates of highly hydrophobic compounds

(3) Problem of waste disposal and recycling

(4) Increase in system complexity

(5) Cost increase to guarantee safety

(6) Clotting of biomass

Organic solvent tolerant bacteria can expand the usability for bioconversion in the presence of a wide range of the solvents and enhance the productivity levels. Many two-phase biotransformation systems using organic solvent tolerant microorganisms have been studied. Several selected examples of these bioconversions are listed in Table 2 and some are described below.

Various characteristics of organic solvents employing in the two phase bioconversion system are required to establish an effective biotransformation system. These include favorable distribution coefficient for product and substrate, high selectivity, low emulsion-forming tendency, low aqueous solubility, chemical and thermal stability, favorable properties for product recovery, non-biodegradability, non-hazardous, inexpensive, available in bulk quantity, and bio-compatibility.

There are several approaches to obtaining organic solvent tolerant microorganisms which possess biocatalytic properties available for the two-phase bioconversion system. The approaches described in this section can be roughly divided into two groups as follows: (1) screening of organic solvent tolerant microorganisms which naturally possess useful biocatalytic properties and (2) introduction of genes encoding for useful enzymes into organic solvent tolerant microorganisms. Both of these approaches require broadening of the knowledge of the mechanism of organic solvent tolerance to construct highly efficient bioconversion systems in the presence of organic

solvents.

1-5-1 Steroid bioconversion in the two-phase system

Cholesterol, being a component of eukaryote cell membranes and a precursor of steroid hormones, is typical of insoluble materials. It is usually used as a raw material for bioproduction of valuable steroids in a water-organic solvent two-phase system to improve the conversion reaction rate. Immobilized cells or enzymes are often employed in the bioproduction of cholesterol dissolved in organic solvents at a high concentration. Thus, cholesterol was used to examine the potential advantage of organic solvent tolerant microorganisms in the two-phase system.

A cholesterol-converting microorganism, *Burkholderia cepacia* strain ST-200 was isolated from humus soil with a screening medium overlaid with organic solvents (6). Strain ST-200 exhibited tolerance to various organic solvents such as decane, nonane, cyclooctane, *n*-octane, diphenylmethane, and cyclohexane, but not *p*-xylene. Strain ST-200 grew in a medium overlaid with a 10% (vol/vol) organic solvents containing cholesterol, and effectively converted cholesterol to 6β-hydroperoxycholest-4-en-3-one and cholest-4-ene-3,6-dione (Fig. 1-6 A). The conversion was not effective in a monophasic system in which cholesterol was suspended. This result indicated that a two phase fermentation system using organic solvent tolerant microorganisms is effective for cholesterol bioconversion. The first step of cholesterol oxidation was catalyzed by an enzyme cholesterol oxidase. This enzyme was highly stable in the presence of organic solvents.

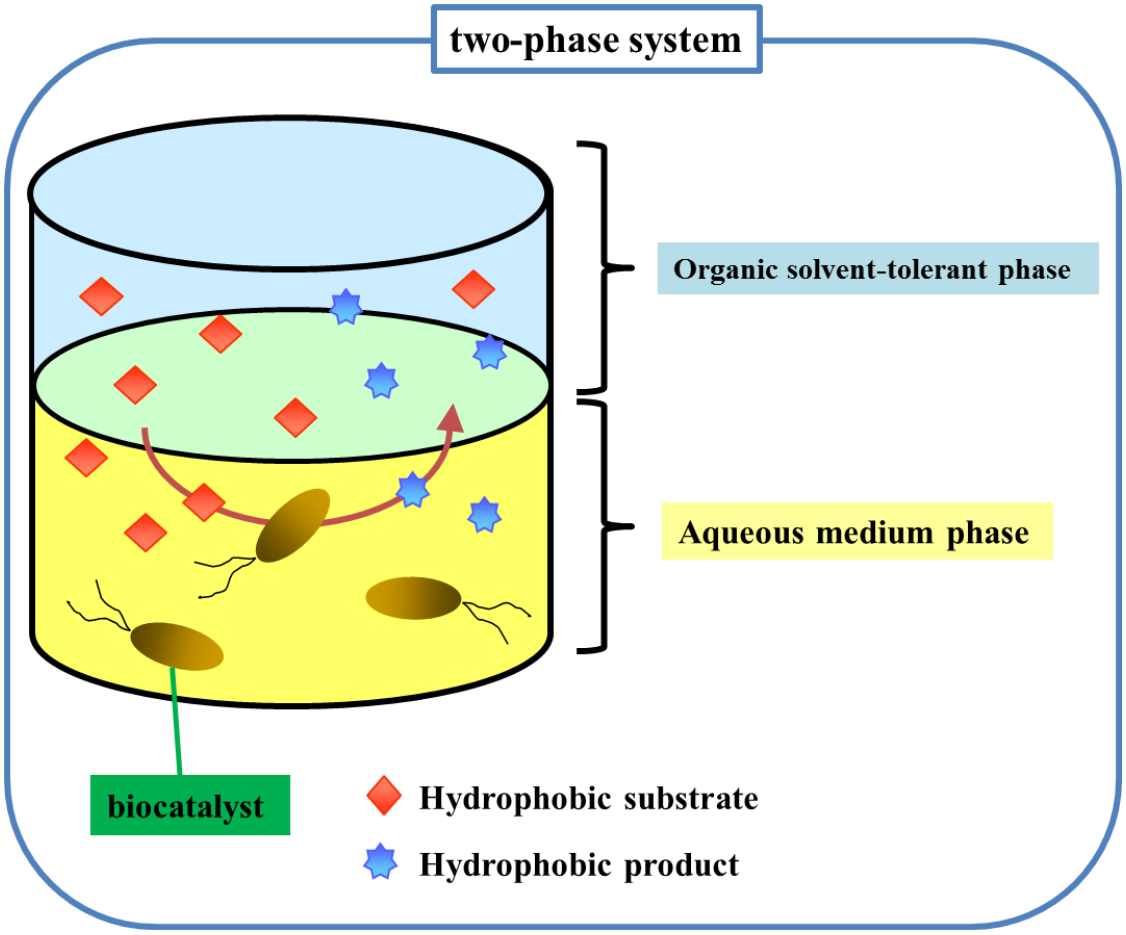

Fig. 1-5 Bioconversion in a two-phase system
Bioconversions of hydrophobic compounds using whole-cell biocatalysts have been applied to in aqueous-organic solvent two-phase systems. The two phase bioconversion system has many advantages.

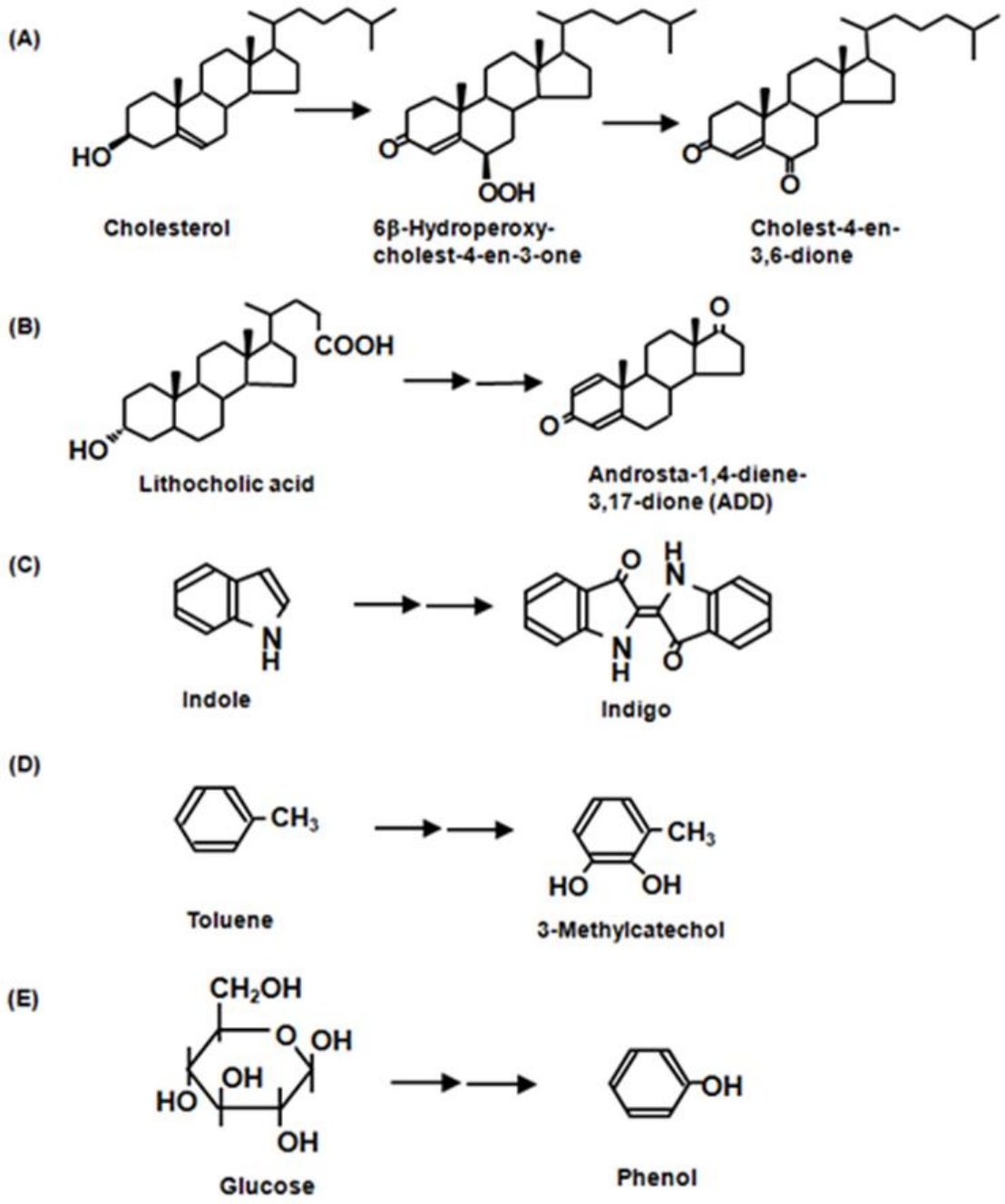

Fig. 1-6 Bioconversion of various compounds in the two phase system (A) Cholesterol bioconversion by *Burkholderia cepacia* strain ST-200, (B) Steroid hormone precursors production from lithocholic acid by *Pseudomonas putida* strain ST-491, (C) Indigo production from indole by *Acinetobacter* sp. ST-550, (D) 3-Methylcatechol production from toluene by *Pseudomonas putida* S12, (E) Phenol production from glucose by *Pseudomonas putida* S12.

Thus, organic solvent tolerant bacteria often produce organic solvent tolerant enzymes.

Cleavage of the C-17 side chains of steroids is crucial for the production of steroid hormones. The use a two-phase bioconversion system was employed for microbial cleavage of the side chains of organic solvent-insoluble steroids (100). Lithocholic acid and deoxycholic acid have C-24 carboxyl groups on their side chains. These bile acids are insoluble in hydrophobic organic solvents due to the polar carboxyl group and 3-hydroxyl group. When the polar carboxyl groups of lithocholic acid were cleaved and the 3-hydroxyl group of the compound was oxidized by microorganisms in the two phase system, more hydrophobic steroid hormone precursors such as androsta-4-dien-3,17-dione (AD) and androsta-1,4-diene-3,17-dione (ADD) were formed and extracted into the organic solvent phase (Fig. 1-6 B). Therefore, the two phase system can be used for an extractive bioconversion of hydrophobic steroid hormone precursors from polar bile acids. An *Pseudomonas putida* strain ST-491 isolated from humus soil was able to convert bile acids to steroid hormone precursors in the presence of cyclooctane. Strain ST-491 was tolerant to cyclooctane, diphenyl ether, *n*-hexane, or *p*-xylene. The time course of steroid hormones production by strain ST-491was examined. In the absence of solvent, strain ST-491 catabolized approximately 30% of the substrate as a carbon source and transiently accumulated ADD in an amount corresponding to 5% of the substrate added. When 20% (vol/vol) diphenyl ether containing 0.5% (wt/vol) lithocholic acid was added to the medium, 60% of the substrate was converted to 17-ketosteroids (AD and ADD) or a 22-aldehyde steroid

(pregne-1,4-dien-3-on-20-al) (PDOA). The amounts of the products were responsible for 45% (ADD), 10% (PDOA), and 5% (AD) of the substrate, respectively. These results showed that diphenyl ether facilitates catabolism from lithocholic acid to ADD rather than suppressing the assimilation of ADD. In addition, these results indicated that a two phase fermentation system can be used not only for bioconversion of a hydrophobic compound but also for that of a compound with low solubility in organic solvents.

1-5-2 Bioproduction of textile dye in the two-phase system

Indigo is one of the world's largest-selling textile dyes used on cotton and wool fabrics. This dye was traditionally produced from plants of the genus *Indigofera*. Plant-derived indigo has been replaced by synthetic indigo in the textile industry. Several attempts have been made to produce indigo from indole by microorganisms expressing monooxygenase or dioxygenase (Fig. 1-6 C) (25, 65). One obstacle to the production of indigo is the toxicity of the substrate indole to the producers. The concentration of indole in the medium must be kept low to avoid the toxic effect (23). However, the substrates are readily consumed by the microorganisms when little indole is supplied. Thus, indigo formation is not very effective. Indole is more soluble in several kinds of organic solvents than in water. Therefore, the two-phase bioconversion system seemed to be useful for the microbial production of indigo from indole. *Acinetobacter* sp. strain ST-550 isolated from humus soil samples, effectively produced indigo from indole in the two phase system (26). ST-550 produced a slight amount of indigo (less than 0.1 mg/ml) when grown in the presence of

indole at concentrations of 0.05 to 0.3 mg/ml. However, ST-550 effectively produced indigo when ST-550 was grown in the presence of a large volume of diphenylmethane and a high level of indole. One of the optimized conditions for indigo production was that ST-550 was grown in 3 ml of a medium containing 0.3 ml of diphenylmethane and 2.7 mg of indole. Under the condition, ST-550 produced 0.88 mg of indigo (292 μg/ml-medium).

Genes involved in the conversion of indole to indigo in strain ST-550 were identified and cloned (24). These genes were identical to those coding for the multicomponent phenol hydroxylase from *Acinetobacter calcoaceticus* NCBI8250. The genes were introduced into *E. coli* JA300 and its cyclohexane-tolerant mutant OST3410, and the production of indigo in the two-phase system was examined with the resulting recombinants. OST3410 recombinant produced 52 μg indigo/ml of the medium in the presence of diphenylmethane. This productivity was 4.3-fold higher than that of JA300 recombinant.

1-5-3 3-Methylcatechol production from toluene in the two-phase system

Catechol and its derivatives have been used in the manufacture of synthetic flavours such as vanillin, and precursors for pharmaceutical production. They are difficult to synthesize chemically (37).

A solvent-tolerant *Pseudomonas putida* S12 efficiently produced 3-methylcatechol from toluene using the solvent-tolerant in an aqueus-octanol two -phase system (Fig. 1-6 D) (106). *P. putida* strain S12 lacks ability to metabolize toluene. This strain was endowed by introduction

of the genes from *P. putida* F1 coding for toluene dioxygenase, *todC1C2BA*, and *cis*-toluene dihydrodiol dehydrogenase, *todD*, to produces 3-methylcatechol from toluene. The maximum concentration of 3-methylcatechol was increased two-fold by the use of the two-liquid medium-octanol system.

P. putida MC2 produced 3-methylcatechol from toluene by in the presence of 1-octanol (36). The strain originally contained a toluene degradation pathway. This native pathway was mutated to stop further enzymatic degradation of the metabolite from toluene. Additional sets of *todC*1*C*2*BAD* genes for 3-methylcatechol production were introduced into this strain. As the result of two-phase bioconversion system using the recombinant, the production of 3-methylcatechol increased compared to that in aqueous media without any solvent.

1-5-4 Phenol bioproduction from glucose in the two-phase system

Bioproduction of chemicals from natural renewable resources received considerable attention in recent times. These processes are possible to reduce fossil resources dependency and maintain closed carbon cycles. A solvent-tolerant *P. putida* S12 effectively producing phenol from glucose by introducing the *tpl* gene from *Pantoea agglomerans*, encoding tyrosine phenol lyase, into the solvent-tolerant strain *P. putida* S12 (Fig. 1-6 E) (107). In a fed-batch process, the productivity was limited by accumulation of 5 mM phenol in the medium. This toxicity was overcome by use of octanol as an extractant for phenol in a biphasic medium-octanol system. This approach

resulted in the accumulation of 58 mM phenol in the octanol phase, and there was a two-fold increase in the overall production compared to a single-phase fed batch.

1-6 Bioremediation

Organic solvent tolerant microorganisms are attractive also for bioremediation decomposing pollutants such as crude oil, aromatic hydrocarbons and sulfur compounds.

Flavobacterium sp. strain DS-711 isolated from deep-sea mud samples showed halotolerant growth and tolerance to various organic solvents such as benzene, toluene and *p*-xylene (63). Strain DS-711 effectively degraded crude oils and various *n*-alkanes. A benzene-, cyclohexane- and *n*-hexane-tolerant *Bacillus* sp. strain DS-1906 also isolated from the deep sea degraded polyaromatic compounds such as naphthalene, fluorine, phenanthrene, anthracene, pyrene, chrysene, and 1,2-benzopyrene in a liquid *n*-hexane-medium two phase system (1).

Sulfur oxides, one of the air pollutants, are generated by the combustion of sulfur-containing fossil fuel. Biodesulfurization is an attractive alternative to remove the sulfur in a petroleum-refining process. The derivatives of dibenzothiophene (DBT) and benzothiophene are the most abundant heterocyclic compounds in petroleum. A benzene-, toluene- and *p*-xylene-tolerant *Bacillus* sp. strain DS-994 isolated from the deep sea was tolerant to various organic solvents grew in a liquid medium overlaid with model petroleum oil containing DBT and degraded DBT dissolved in the oil

(64).

A solvent-tolerant desulfurizing bacterium, *Pseudomonas putida* A4, was constructed by introducing the biodesulfurizing gene cluster *dszABCD* from *Rhodococcus erythropolis* XP, into the solvent-tolerant strain *P. putida* Idaho (101). Strain A4 effectively degraded DBT in the presence of *p*-xylene.

1-7 Application of efflux pump in nano device

Antibiotics are difficult to selectively remove from surface waters by present treatment methods. The AcrAB-TolC efflux pump is a principal multidrug exporter in *E. coli* and the pump has evolved the ability to discriminately expel antibiotics and other noxious agents via proton and ATP driven pathways. Vikram Kapoor and David Wendell reported light-dependent removal of antibiotics by engineering the bacterial efflux pump AcrB into a proteovesicle system (105). They have created a chimeric protein with the requisite proton motive force by coupling AcrB to the light-driven proton pump Delta-rhodopsin (dR) via a glycophorin A transmembrane domain. This creates a solar powered protein material capable of selectively capturing antibiotics from bulk solutions. Using environmental water and direct sunlight, the AcrB-dR vesicles removed almost twice as much antibiotic as the treatment standard, activated carbon. Altogether, the AcrB-dR system provides an effective means of extracting antibiotics from surface waters as well as potential antibiotic recovery through vesicle solubilization.

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Chapter 2

Contributions of mutations in *acrR* and *marR* genes to organic solvent tolerance in *Escherichia coli*

2-1 Abstract

A number of solvent-efflux pumps involved in organic solvent tolerance have been identified in *Pseudomonas* spp. and *Escherichia coli.* The AcrAB-TolC efflux pump plays an important role in organic solvent tolerance in *E. coli.* Mutations in regulatory genes such as *marR*, *soxR*, and *acrR* are known to enhance the expression level of the AcrAB-TolC pump. In this study, we identified these mutations in organic solvent tolerant *E. coli*. Eight cyclohexane-tolerant *E. coli* JA300 mutants were isolated, and mutations in *marR*, *soxR*, and *acrR* in these mutants were examined. Every mutant carried a mutation in either *marR* or *acrR*. Among all mutants, strain CH7 carrying a nonsense mutation in *marR* (named *marR109*) and an insertion of IS5 in *acrR*, exhibited the highest organic solvent tolerance levels. To examine the involvement of these mutations in improving organic solvent tolerance, they were introduced into the *E. coli* JA300 chromosome. Consequently, JA300 mutants carrying *acrR*::IS5, *marR109*, or both were constructed and named JA300 *acrR*IS, JA300 *marR*, or JA300 *acrR*IS *marR*, respectively. The organic solvent tolerance levels of these mutants were increased in the following order: JA300 < JA300 *acrR*IS < JA300 *marR* < JA300 *acrR*IS *marR*. JA300 *acrR*IS *marR* formed colonies on an agar plate overlaid with cyclohexane and *p*-xylene (6:4 vol/vol mixture). The organic

solvent tolerance level and AcrAB-TolC efflux pump-expression level in JA300 *acrR*IS *marR* were similar to those in CH7. Thus, it was shown that the synergistic effects of mutations in only two regulatory genes (*acrR* and *marR*) can remarkably elevate the level of organic solvent tolerance in *E. coli*.

2-2 Introduction

Whole-cell biocatalysts in two phase systems containing an organic solvent have a broad spectrum of applications for the production of various fine chemicals (14, 36, 37). Two phase systems are employed in order to solubilize reactants and products but also to deal with the problem of toxic substrates and products. In addition, the use of a second organic phase improves productivity levels and product recovery, unlike the case with conventional media whose substrate solubility is poor. One of the main limitations in the application of whole-cell biocatalysts in two-phase systems is the instability of biocatalysts due to the toxicity of organic solvents toward the cells. When microorganisms are incubated in the presence of a large amount of an organic solvent, the extent of growth inhibition is inversely correlated with the log P_{OW} of the solvent (15). Hydrophobic organic solvents with a log P_{OW} of 2 to 5 bind to the cells and disrupt the cell membrane (5, 40).

Various mechanisms underlying microbial tolerance and responses to solvents have been revealed by the genetic, physiological and biochemical characterization of organic solvent tolerant bacteria such as *Pseudomonas* species and *E. coli* mutants (7, 14, 25-27, 33, 38, 39, 41). Until now, more is

known about how cells respond to organic solvents, but less about how to develop tolerant strains.

Energy-dependent efflux pumps belonging to the resistance/nodulation/cell division (RND) family (29) are important for the maintenance of solvent tolerance in Gram-negative bacteria (11, 17, 21, 32, 42). In *E. coli*, the AcrAB-TolC efflux pump belonging to the RND family has been shown to provide intrinsic tolerance to organic solvents (42). This pump enhances the release of solvents intracellularly accumulated in *E. coli* cells. *acrAB* and *tolC* are *marA/soxS/rob* regulon genes (Fig. 1-4) (10). MarA and SoxS proteins are transcriptional activators belonging to the AraC/XylS family (2). These activators control the expression of *marA/soxS/rob* regulon genes. MarA and SoxS are transcriptionally regulated. Transcription of the *marRAB* is repressed by MarR, whereas it is autoactivated by MarA. *soxS* transcription is repressed by SoxR and enhanced by the activated form of SoxR after exposure to superoxides or nitric oxide (30). Mutations in *marR* or *soxR* were suggested to enhance the expression level of the AcrAB-TolC efflux pump (4, 19, 28, 34). In addition, *acrAB* expression is modulated locally by the repressor AcrR (23). Thus, mutations in *acrR* can lead to the enhanced expression of AcrAB. Mutations conferring a multidrug resistance phenotype have been found in the genes *marR*, *soxR*, and *acrR* among clinical and veterinary *E. coli* isolates (13, 16, 19, 44, 45). Some of those studies suggested that organic solvent tolerance is correlated with these mutations in these isolates (19, 44). However, the extent to which these mutations contribute to organic solvent tolerance has not been clarified

because *E. coli* isolates used in these studies had a variety of genetic backgrounds. In addition, the synergistic effects of these mutations on organic solvent tolerance were ambiguous. To clarify the effects of mutations on the tolerance phenotype, it is necessary to reconstruct selected mutations in one type of strains in various combinations.

In this study, we isolated organic solvent tolerant *E. coli* mutants and identified mutations in regulatory genes such as *marR*, *soxR*, and *acrR*. Among these mutants, we selected the one that exhibited the highest organic solvent tolerance and investigated the contributions of each identified mutation to organic solvent tolerance. As a result, it was clarified that the *E. coli* strain can acquire high-level organic solvent tolerance due to mutations in only two regulatory genes (*marR* and *acrR*) by reconstructing these mutations in a parent strain.

2-3 Materials and methods

2-3-1 Materials, media, and culture conditions

The organic solvents used were of the highest quality available (Wako Pure Chemical Industries, Osaka, Japan). The purity of these solvents is more than 98%. The organisms were grown aerobically at 30°C in LBG medium consisting of 1% Bacto Tryptone (Difco Laboratories, Detroit, MI), 0.5% Bacto Yeast Extract (Difco), 1% NaCl, and 0.1% glucose. This medium supplemented with 10 mM MgSO4 (LBGMg medium) (31) was also used. The LBGMg medium was solidified with 1.5% (wt/vol) agar. Ampicillin (50 μg/ml) or kanamycin (50 μg/ml) was added to the medium when necessary.

The organisms were also grown in LB medium, which is identical to LBG medium except that glucose is omitted.

2-3-2 Bacterial strains and plasmids

E. coli K-12 derivatives used to evaluate solvent tolerance are summarized in Table 2-1. Strain JW0452, a BW25113-based *acrA*::KmR (kanamycin resistant), was supplied by the National Bio-Resource Project (NIG, Mishima, Japan): *E. coli* (9).

Table 2-1 Bacterial strains and plasmids used

E. coli strain	Genotype	Reference
JA300	F⁻ *thr leuB6 trpC1117 thi rpsL20 hsdS*	(33)
JA300 *acrR*::IS	Same as JA300, but with *acrR*::IS*5*	This study
JA300 *marR*	Same as JA300, but with *marR109*	This study
JA300 *acrR*::IS *marR*	Same as JA300, but with *acrR*::IS*5* and *marR109*	This study
JA300⊿*acrA*	Same as JA300, but with *acrA*::Km^R	This study
JA300⊿*acrA* *acrR*::IS *marR*	Same as JA300, but with *acrA*::Km^R, *acrR*::IS*5* and *marR109*	This study
BW25113	$lacI^q$ $rrnB_{T14}$ $lacZ_{WJ16}$ *hsdR*514 $araBAD_{AH33}$ $rhaBAD_{LD78}$	(10)
JW0452	Same as BW25113, but with *acrA*::Km^R	(10)

2-3-3 Isolation of cyclohexane-tolerant mutants of *E. coli* JA300

First, 100 µl of the overnight culture of strain JA300 grown in LBGMg medium was inoculated into 10 ml of fresh LBGMg medium overlaid with 2 ml cyclohexane. The two-phase culture was incubated at 37°C with shaking. Since the growth of cyclohexane-tolerant mutants was observed after 48 h, the aqueous medium phase was spread on LBGMg agar medium. Then, the agar surface was overlaid with a 3-mm-thick layer of cyclohexane. Strains that formed relatively large colonies on the agar after 48 h incubation at 25°C were randomly isolated as cyclohexane-tolerant mutants.

2-3-4 PCR amplification and DNA sequencing of *acrR*, *marR*, and *soxR*

Chromosomal DNA of each bacterial isolate was used as the template for polymerase chain reaction (PCR) amplification. PCR was performed using PrimeSTAR® GXL DNA polymerase (Takara Bio, Kyoto, Japan). The primers used for PCR are listed in Table 2-2. The primer combinations used are as follows: acrR-F and acrR-R for the entire *acrR*; marR-F and marR-R for the entire *marR* and the operator region for *marR*; and soxR-F and soxR-R for the entire *soxR*. The PCR products were purified using the QiaQuick PCR purification kit (Qiagen, Hilden, Germany). Direct cycle sequencing in both directions was performed with the same sets of primers.

Table 2-2 Primers used in this study

Primer	Sequence (5′ to 3′)	Positions[a]
acrR-F	AAACCCATTGCTGCGTTTAT	-90 to -71 bp of *acrR*
acrR-R	AAACCGCAAGAATATCACGA	+711 to +730 bp of *acrR*
marR-F	CTGTTCATGTTGCCTGCCAG	-321 to -302 bp of *marR*
marR-R	CAGTCCAAAATGCTATGAATGG	+482 to +503 bp of *marR*
soxR-F	TTTCTGATGGGACATAAATCTGCC	-100 to -77 bp of *soxR*
soxR-R	TGTGTTGACGTCGGGGGAAA	+537 to +556 bp of *soxR*
acrA-F	CCAATTTGAAATCGGACACTCG	-32 to -11 bp of *acrA*
acrA-R	GCATGTCTTAACGGCTCCTG	+1200 to +1219 bp of *acrA*
acrR-rpsL-neo-F	*GCTTCAGGATAATCCCGCTAACTTGAGGACGAACTTCTGCGATCCGGTAG*GGCCTGGTGATGATGGCGGGATCG	-376 to -327 bp of *acrR* (indicated by italics), and -138 to -115 bp of *rpsL* in the *rpsL-neo* cassette (underlined)
acrR-rpsL-neo-R	*GCGATCGATTTTATCGAGGGTGGCTAATGTATCTGTCAGATCCTGCTGCA*TCAGAAGAACTCGTCAAGAAGGCG	+974 to +1023 bp of *acrR* (indicated by italics), and +750 to +773 bp of *neo* in the *rpsL-neo* cassette (underlined)
marR-rpsL-neo-F	*AAACCGATAAACGCGACGATTAAGCCGCCTGCAATTCGCAGACCGGGAAT*GGCCTGGTGATGATGGCGGGATCG	-477 to -428 bp of *marR* (indicated by italics), and -138 to -115 bp of *rpsL* in the *rpsL-neo* cassette (underlined)
marR-rpsL-neo-R	*AAGAGAATAAGCGCAGCTGCTATTGCGGATGAAAGTGGTTTCATGATTGC*TCAGAAGAACTCGTCAAGAAGGCG	+863 to +912 bp of *marR* (indicated by italics), and +750 to +773 bp of *neo* in the *rpsL-neo* cassette (underlined)
Repair-acrRIS-F	TGATCGTACTCTTGCTTACTGAT	-575 to -553 bp of *acrR*
Repair-acrRIS-R	ATCGTTTTGTGCGTTTTGCAAAT	+1202 to +1224 bp of *acrR*
Repair-marR-F	TTTTCGCCTCCGGTGAATCA	-527 to -508 bp of *marR*
Repair-marR-R	AACTGGCTGCGTGGTTTGTT	+933 to +952 bp of *marR*

[a]The positions of the primers are relative to the start codon of each gene.

2-3-5 Site-directed point mutations in *E. coli* chromosome

For site-directed mutagenesis, the phage λ-based homologous recombination system (Red/ET counterselection Bac modification kit; GeneBridges, Heidelberg, Germany) was used to introduce an *rpsL-neo* cassette into *acrR* or *marR* of strain JA300 and to subsequently replace the cassette with an appropriate DNA fragment. Linear DNA fragments comprising the *rpsL-neo* cassette for the introduction of the *acrR* and/or *marR* region were obtained by PCR using the *rpsL-neo* template DNA (GeneBridges) as the template. The combinations of primers for the introduction of the *rpsL-neo* cassette were as follows: acrR-rpsL-neo-F and acrR-rpsL-neo-R for *acrR*, and marR-rpsL-neo-F and marR-rpsL-neo-R for *marR* (Table 2-2). These primers contain homology arms consisting of 50 nucleotides upstream and downstream from the targeted region and 24 nucleotides homologous to the *rpsL-neo* cassette. For all amplification, PrimeSTAR HS DNA polymerase (Takara Bio) with high fidelity was used. The *acrR* or *marR* region into which the *rpsL-neo* cassette was introduced was replaced with corresponding DNA fragments containing a mutated gene of *acrR* or *marR* from strain CH7. The DNA fragments for the replacement of the *rpsL-neo* cassette were obtained by PCR using chromosomal DNA prepared from strain CH7 as the template. The combinations of primers for replacement of the *rpsL-neo* cassette are as follows: Repair-acrRIS-F and Repair-acrRIS-R for *acrR*, and Repair-marR-F and Repair-marR-R for *marR*. Transformations of cells by the introduction of linear DNA fragments for recombination were performed by electroporation according to the protocol

recommended by the technical manual of the Bac modification kit (Gene Bridges). Briefly, the *E. coli* strain carrying pRed/ET (Gene Bridges) was cultivated in 1.4 ml of LB medium at 30°C. At an absorbance of 0.3 (600 nm), freshly prepared L-arabinose was added (0.35% wt/vol, final concentration) to the culture inducing *redyβa*/*recA* expression, and expression was continued at 37°C. After 1 h, the cells were harvested by centrifugation, washed with 10% (vol/vol) ice-cold glycerol, and resuspended in a final volume of 30 μl in 10% (vol/vol) ice-cold glycerol. DNA fragments (100 to 200 ng) were then added to the sample. Subsequently, the sample was transferred to electroporation cuvettes, and electroporation was carried out with an Electroporator 2510 (Eppendorf, Hamburg, Germany) at 1350 V, 10 μF, and 600 Ohms. The cells were immediately removed from the cuvettes by mixing with 1 ml LB medium, and then were incubated at 37°C for 2 h. The cells were plated on LB agar containing the appropriate antibiotics.

Recombination events were confirmed by PCR and DNA sequencing of the *acrR* and *marR* genes. The combinations of primers used are as follows: acrR-F and acrR-R for *acrR*, and marR-F and marR-R for *marR*.

2-3-6 Disruption of *acrA* in strain JA300 and JA300 *acrR*::IS *marR*

JA300Δ*acrA* and JA300Δ*acrA acrR*::IS *marR* were constructed by P1 transduction of kanamycin resistance, with strain JW0452 as the donor. Disruption of the gene was confirmed by PCR analysis using chromosomal DNA prepared from JA300Δ*acrA* or JA300Δ*acrA acrR*::IS *marR* as the template. The combination of primers used was acrA-F and acrA-R (Table

2-2). Consistent with the expected values, the size of the amplified product (*acrA*) from JA300 was 1,252 bp, and those from kanamycin-resistance cassette transductants were approximately 1.5 kb.

2-3-7 Measurement of the organic solvent tolerance of *E. coli*

Cultures of E. coli strains in LBGMg medium (optical density at 660 nm [OD660], 0.4 to 0.6) were diluted with 0.8% saline by serial 10-fold dilutions. Each suspension was plated on LBGMg agar. The agar surface was overlaid with a 3-mm-thick layer of an organic solvent. The approximate frequency at which the cells formed colonies on the agar was estimated after 48 h incubation at 25°C.

2-3-8 Quantitation of organic solvent accumulation in *E. coli* cells

The organic solvent accumulation in *E. coli* cells was quantitated as described previously (12). An organic solvent was added to a suspension of *E. coli* cells harvested during the late exponential phase of growth (OD660, 1.5 to 2.0). The suspension was centrifuged after incubation with the solvent for 30 min. After separation from the medium layer, the solvent layer was removed by aspiration. The medium was disposed of by decanting. The cell pellet was recovered and suspended in 1.0 ml of 0.9% NaCl–10 mM $MgSO_4$. A 0.5 ml portion of the suspension was then transferred to an Eppendorf tube, and the remaining portion was kept to measure the protein content. The 0.5 ml cell suspension was extracted with 2.0 ml of CHCl3 by vigorous shaking for 90 min at 25°C in a shaker (Handless shaker SHK-COCK; Asahi

Technoglass, Tokyo, Japan). The amount of organic solvent in the $CHCl_3$ extract was measured using a gas chromatography–mass spectrometry apparatus (GCMS-QP2010 Plus; Shimadzu, Kyoto, Japan) with an Rtx®-624 column (30 m by 0.25 mm inside diameter, 1.4 μm film thickness; Restek, PA, USA). The column was eluted with helium gas at a flow rate of 1.69 ml/min. The inlet was set at 150°C, and the oven was programmed as follows: 50°C for 5 min, then increasing by 5°C per minute to 100°C. The total ion chromatogram of an organic solvent was detected with a mass selective detector.

2-3-9 Protein content

Protein content was measured by the method of Lowry et al. (22).

2-3-10 Antibodies against AcrA, AcrB, and TolC

Antibodies against AcrA were obtained as described previously (12). Polyclonal antibodies against AcrB and TolC were raised against synthetic peptides corresponding to regions of AcrB and TolC. These antibodies contained an N-terminal cysteine (C + in the peptides shown below) for the conjugation of keyhole limpet hemocyanin. The peptide sequences were as follows: a synthetic peptide for anti-AcrB antibodies, C + KNEDIEHSHTVDHH (corresponding to residues 1036 to 1049) and a peptide for anti-TolC antibodies, C + ARTTTSNGHNPFRN (corresponding to residues 480 to 493). The conjugated peptides were injected into rabbits, and polyclonal antibodies were purified from serum using a Melon gel

immunoglobulin G spin purification kit (Thermo Fisher Scientific, Rockford, IL, USA). These antibodies were used to detect AcrA, AcrB, and TolC in the immunoblotting analyses of this study.

2-3-11 Immunoblotting analyses

E. coli was grown in LBGMg medium. The cells were harvested during the exponential phase of growth (OD660, 0.6) by centrifugation (5,000 × g for 10 min at 4°C). The cells were suspended in cold 10 mM Tris–HCl buffer (pH 8.0) and broken by sonication in an icewater bath. Ten micrograms of total cell lysate protein in the supernatant was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12.5% separating gel (43). The gels were then transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA), electrophoretically by the application of 50 V for 30 min in Tris-glycine-methanol buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol [pH 8.3]). The membrane was blocked overnight at room temperature in Tris-buffered saline (TBS; 0.15% NaCl, 10 mM Tris–HCl, pH 7.4) containing 3% gelatin, washed three times in wash buffer (0.05% Tween 20 in TBS), and hybridized at room temperature with the relevant antibody. The membrane was then probed for 1 h with goat anti-rabbit horseradish peroxidase (Bio-Rad, Hercules, CA, USA). The bands were visualized by use of an alkaline phosphatase color development kit (Bio-Rad). The AcrA, AcrB, and TolC expression levels were quantified by gel analysis software UN-SCAN-IT (Silk Scientific, Orem, UT, USA).

2-3-12 Antibiotic susceptibility

The minimum inhibitory concentrations (MICs) of various antibiotics were determined by a sequential dilution method (8). LBG medium liquid cultures containing different concentrations of antibiotic and freshly grown 10^3 cells of the tested *E. coli* strain were incubated at 37°C for 16 h. The lowest concentration of antibiotic which completely inhibited growth was defined as the MIC (8)

2-4 Results and discussion

2-4-1 Isolation of cyclohexane-tolerant mutants of *E. coli* JA300

E. coli K-12 strain JA300 is tolerant to an organic solvent with a $\log P_{OW}$ value of more than 4 and is highly sensitive to cyclohexane ($\log P_{OW}$, 3.4) (7). Cyclohexane-tolerant mutants that formed colonies on the LBGMg agar medium overlaid with cyclohexane were isolated from strain JA300. Spontaneous cyclohexane-tolerant mutants from strain JA300 appeared on the LBGMg agar medium overlaid with cyclohexane at a frequency of 1.6×10^{-6}. In the present study, to efficiently isolate mutants with high-level organic solvent tolerance, the parent strain JA300 was precultured in the LBGMg liquid medium overlaid with cyclohexane. The viable cell density in the preculture increased to 2.2×10^5 /ml after 48 h incubation. The preculture was spread on the agar medium, and then the agar surface was overlaid with cyclohexane. After 48 h, mutants appeared on the agar medium with cyclohexane at a frequency of 5.0×10^{-3}. The organic solvent tolerances of about 100 isolates randomly selected were investigated by

measuring colony-forming efficiencies on an agar plate overlaid with cyclohexane. As a result, eight mutants (CH1 to CH8) exhibiting relatively high colony-forming efficiencies on the agar medium with cyclohexane were selected and used in further experiments.

2-4-2 Organic solvent tolerances of the isolated mutants

The organic solvent tolerances of the mutants (CH1 to CH8) were investigated by measuring the colony-forming efficiency of each mutant on an agar plate overlaid with pure cyclohexane, cyclohexane and *p*-xylene (8:2 vol/vol mixture) and a mixture of cyclohexane and p-xylene (7:3 vol/vol mixture) (Fig. 2-1). *p*-Xylene ($\log P_{OW}$, 3.1) shows higher toxicity to cells than cyclohexane. All strains formed colonies in all spots on the plate without any solvent. The parent strain JA300 did not form any colony in the presence of cyclohexane and the solvent mixture. All mutants formed colonies in the presence of cyclohexane, although the colony-forming efficiencies differed among the mutants. Strains CH1, CH3, CH4, and CH7 exhibited relatively high organic solvent tolerance in the presence of cyclohexane. Strains CH2, CH5, CH6, and CH8 did not form any colonies in the presence of the solvent mixture. On the other hand, strain CH7 formed colonies even in spots containing 10^3 cells in the presence of the solvent mixture and therefore showed the highest colony-forming efficiency among all isolates.

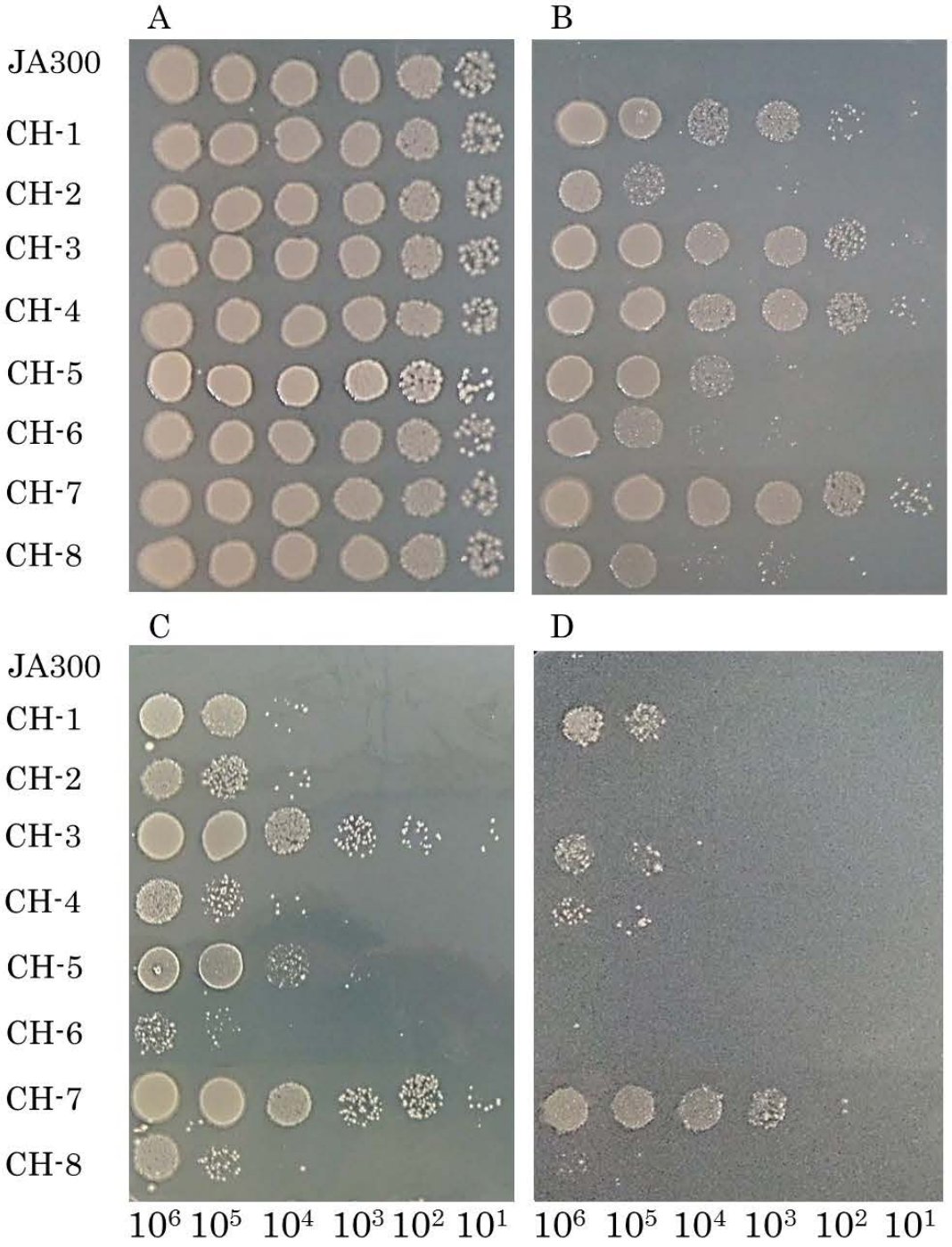

Fig. 2-1 Organic solvent tolerances of the isolated mutants
Colony-forming efficiency of organic solvent-tolerant mutants from *E. coli* JA300 on LBGMg agar medium in the absence of an organic solvent (A) and in the presence of cyclohexane (B), cyclohexane and *p*-xylene (8:2 vol/vol mixture)(C), or cyclohexane and *p*-xylene (7:3 vol/vol mixture) (D).

2-4-3 Identification of mutations in *acrR*, *marR*, and *soxR*

The nucleotide sequences of entire *acrR*, *marR*, and *soxR* genes in organic solvent tolerant mutants were determined by DNA sequencing to identify mutations in these genes. The DNA sequences were analyzed by comparison with those of the JA300 parent strain. These mutations are summarized in Table 2-3. The sequences of *acrR*, *marR*, and *soxR* genes in strain JA300 were identical to those present in the *E. coli* K-12 strain MG1655 genome deposited in GenBank (accession number NC_000913.2). In fluoroquinolone-resistant clinical and veterinary isolates of *E. coli*, a number of mutations were found in *soxR* as well as *marR* and *acrR* (19, 44, 45). None of the mutants carried a mutation in *soxR*. Seven of the eight cyclohexane-tolerant mutants carried four different mutations in *marR*. Among these seven *marR* mutants, three mutants (CH1, CH3, and CH4) had point mutations causing an amino acid substitution. These mutations were G116C (CH1), L78M (CH3), and R94L (CH4). Four strains (CH5, CH6, CH7, and CH8) had the same mutation, which would cause premature termination of the *marR* translation. This was caused by a nonsense codon (E109 → TAA stop). This mutation was named *marR109*. These MarR mutations lie within the region spanning amino acids 61 to 121 in MarR, which are required for DNA binding activity (3). The L78 and R94 residues are highly conserved amino acids in many homologs, although the amino acid at the position of G116 is not conserved (3). The marR109 mutation led to the truncation of 35 C-terminal amino acids in MarR. A previous study showed that the C-terminal region contributes to dimer formation (24). In any case, the MarR mutations found in the present study are considered to lead to the loss of repressive function. Three mutants (CH1, CH2, and CH7) carried mutations in *acrR*. Strain CH2 had a mutation only in *acrR*. This mutation was found in the translation initiation codon (M1I). The mutation in the translation initiation codon (M1I) will cause the complete inhibition of AcrR translation. The other two mutants (CH1 and CH7) each carried mutations in both *marR* and *acrR*. Strain CH1 had a point mutation altering one amino acid residue (A41D) in *acrR*. The A41 residue positioned within the helical region (α3) forms part of a typical helix-turn-helix motif involved in DNA binding (20). Thus, the A41D mutation will abolish AcrR's repressive activity. Strain CH7 contained an 1195-bp insertion sequence (IS5) (35) integrated within *acrR* (Fig. 2-2). This sequence was inserted at 220 bp downstream from the

initiation codon of the *acrR* gene, accompanied by the doubling of the tetramer CTAG. The *ins5A* promoter was oriented in the same direction as the acrAB operon. The alteration of organic solvent tolerances of several *E. coli* mutants by IS integration has been reported. *E. coli* OST4251 became sensitive to n-hexane because IS*2* and IS*5* became integrated upstream from the *imp*/*ostA* gene, which is involved in organic solvent sensitivity (1). The hypersensitivity of an *E. coli acrB* disruptant to organic solvents was suppressed by the integrational activation of the *acrEF* operon with an IS1 or IS2 element (18).

Table 2-3 Mutations in *marR* and *acrR* of cyclohexane-tolerant *E. coli* mutants

strain	*marR*		*acrR*		*soxR*	
	DNA positiona (Codon substitution)	Amino acid substitution	DNA positiona (Codon substitution)	Amino acid substitution	DNA positiona (Codon substitution)	Amino acid substitution
CH1	346 (GGC→TGC)	G116C	122 (GCT→GAT)	A41D		None
CH2		None	3 (ATG→ATA)	M1I		None
CH3	232 (CTG→ATG)	L78M		None		None
CH4	281 (CGC→CTC)	R94L		None		None
CH5	325 (GAA→TAA)	E109→TAA stop		None		None
CH6	325 (GAA→TAA)	E109→TAA stop		None		None
CH7	325 (GAA→TAA)	E109→TAA stop	220	Insertion of IS*5*		None
CH8	325 (GAA→TAA)	E109→TAA stop		None		None

[a]DNA positions of the mutations are relative to the start codon of each gene.

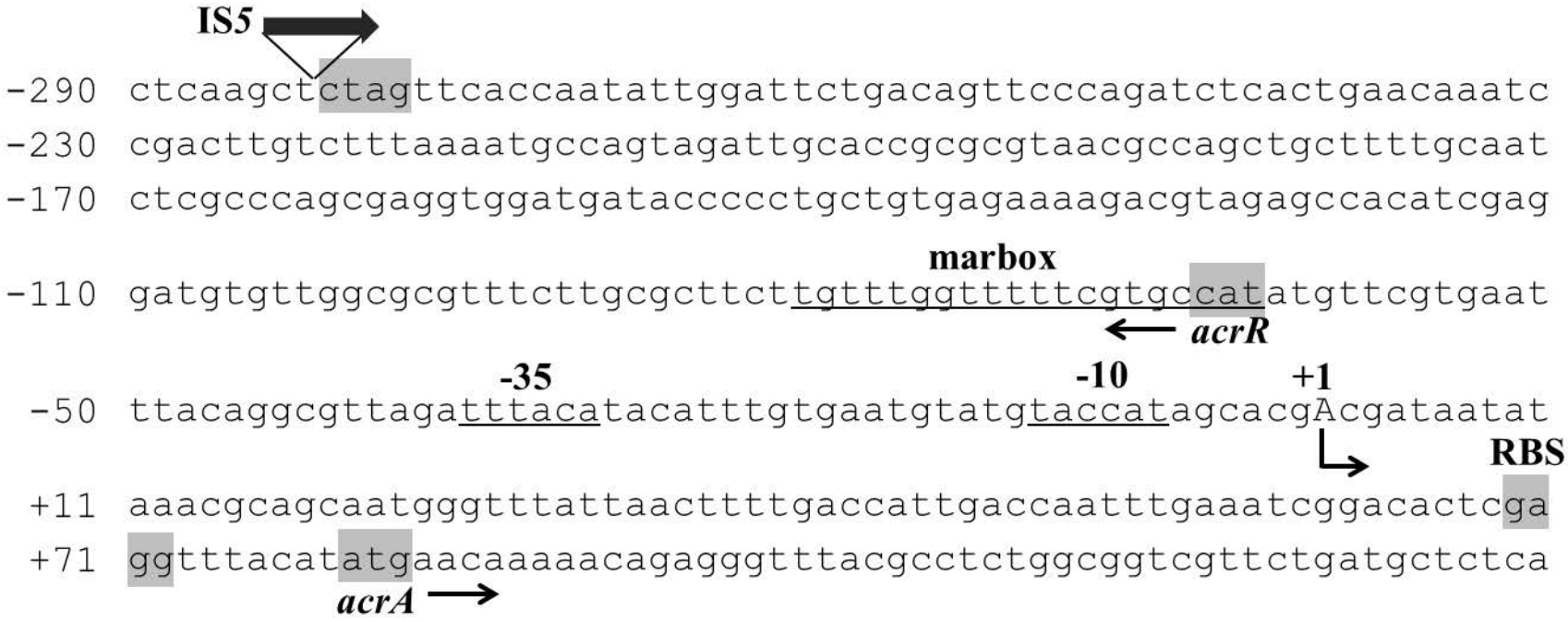

Fig. 2-2 Nucleotide sequence of the *acrAB* regulatory region.
The transcription initiation site (+ 1) of the *acrAB* operon is capitalized and indicated by a bent arrow. The CTAG IS*5* target site, the putative ribosome binding site (RBG) for *acrA*, and the ATG start codons for *acrR* and *acrAB* are shaded. The promoter regions (– 10 and – 35) and marbox are underlined. The bold arrow above the sequence indicates the insertion site of IS*5*.

2-4-4 Organic solvent tolerances of mutants carrying mutations in *marR* and/or *acrR* derived from strain CH7

Strain CH7 had a nonsense mutation in *marR* and an insertion of IS5 in *acrR*. To clarify the involvement of these mutations in organic solvent tolerance, the *marR* and/or *acrR* regions were introduced into the *E. coli* JA300 chromosome by site-directed mutagenesis using λ red-mediated homologous recombination. Consequently, JA300 mutants carrying *acrR*::IS5, *marR*109, or both mutations were constructed and named JA300 *acrR*IS, JA300 *marR*, or JA300 *acrR*IS *marR*, respectively. The colony-forming efficiencies of these constructed mutants in the presence of organic solvents were compared with those of the parent strain JA300 and strain CH7 (Fig. 2-3). All strains formed colonies in all spots on the plate without any solvent. The parent strain JA300 formed colonies in the spots containing 10^5-10^6 cells in the presence of *n*-hexane (logP_{OW}, 3.9). However, strain JA300 hardly formed colonies on the plate overlaid with pure cyclohexane and did not form any colony on the plate with cyclohexane and *p*-xylene (6:4 mixture). In contrast, the colony-forming efficiencies of the constructed mutants in the presence of the organic solvents were increased in the following order: JA300 *acrR*IS < JA300 *marR* < JA300 *acrR*IS *marR*. JA300 *acrR*IS *marR* exhibited about 10^2- and 10^4-fold higher colony-forming efficiencies than those of JA300 *acrR*IS and JA300 *marR*, respectively, in the presence of cyclohexane. JA300 *acrR*IS and JA300 *marR* did not form any colony on the plate overlaid with the solvent mixture, although JA300 *acrR*IS *marR* formed colonies in spots containing 10^5-10^6 cells in the presence of the solvent mixture. JA300

acrR::IS *marR* showed similar colony-forming efficiencies as strain CH7 in the presence of the solvents tested. The cell growth of JA300, JA300 *acrR*::IS *marR*, and CH7 in the LBGMg liquid medium in the presence of *n*-hexane or cyclohexane was also examined by measuring turbidity (Fig. 2-4). No significant difference was found between the growth of these strains in the absence of organic solvents. The growth of JA300 was highly suppressed by the addition of organic solvents. In contrast, JA300 *acrR*::IS *marR* and CH7 were able to grow in the presence of these solvents, although the growth rates and yields of these strains were partially reduced by the addition of these solvents as compared to that without any solvent.

Organic solvent tolerance levels of various mutants and recombinants from strain JA300 have been investigated by measuring the colony-forming efficiencies of mutants on an LBGMg agar plate overlaid with organic solvents. Overexpression of the *marA* gene has been shown to raise the organic solvent tolerance of *E. coli* (8, 39). JA300 overexpressing the *marA* gene formed colonies in spots containing more than 10^6 cells in the presence of cyclohexane (39). In addition, it was reported that the organic solvent tolerance of strain JA300 significantly improved the double disruptions of *marR* and *proV* (12). JA300Δ*proV*Δ*marR* formed colonies in spots containing more than 10^5 cells in the presence of cyclohexane and thus exhibited higher organic solvent tolerance levels than JA300 overexpressing the *marA* gene. In the present study, JA300 *acrR*::IS *marR* showed 10^4-fold higher colony-forming efficiencies in the presence of cyclohexane than JA300Δ*proV* Δ*marR*.

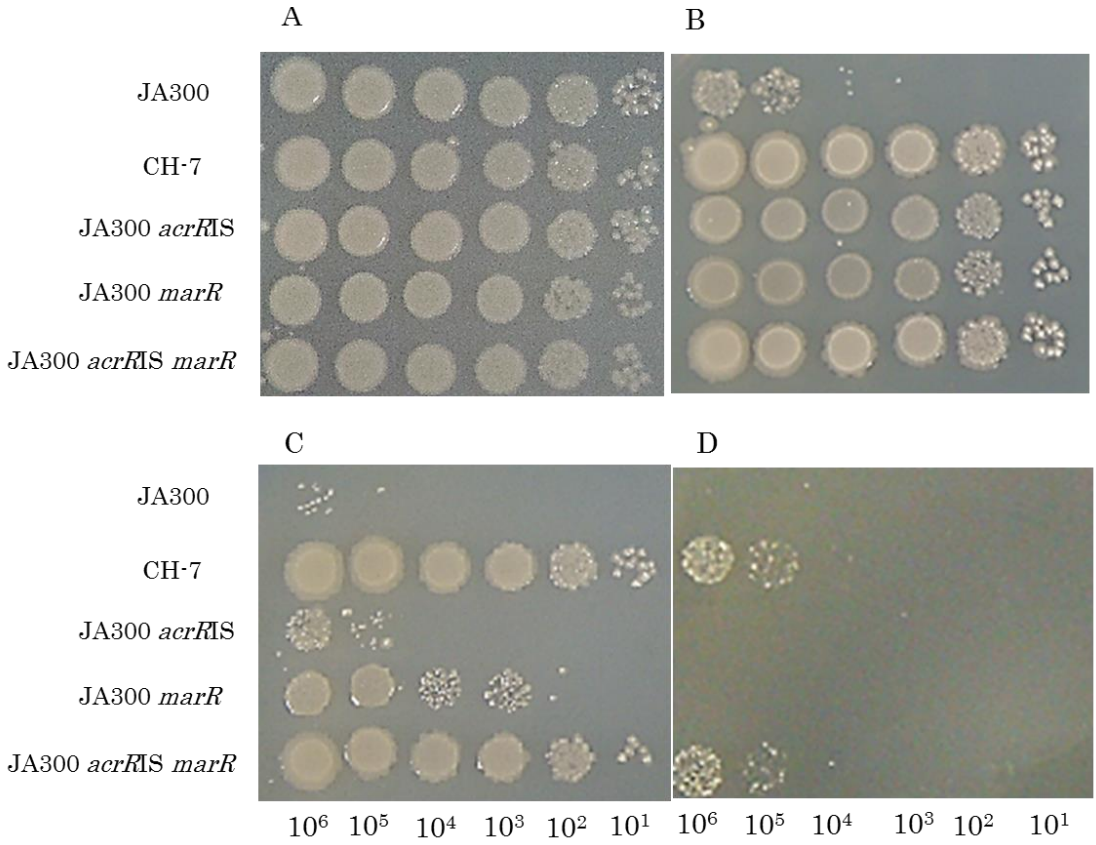

Fig. 2-3 Organic solvent tolerances of mutants carrying mutations in *marR* and/or *acrR* derived from strain CH7

Colony-forming efficiency of *acrR* and/or *marR* mutants on the agar medium in the absence of an organic solvent (A) and in the presence of *n*-hexane (B), cyclohexane (C), or cyclohexane and *p*-xylene (6:4 vol/vol mixture) (D).

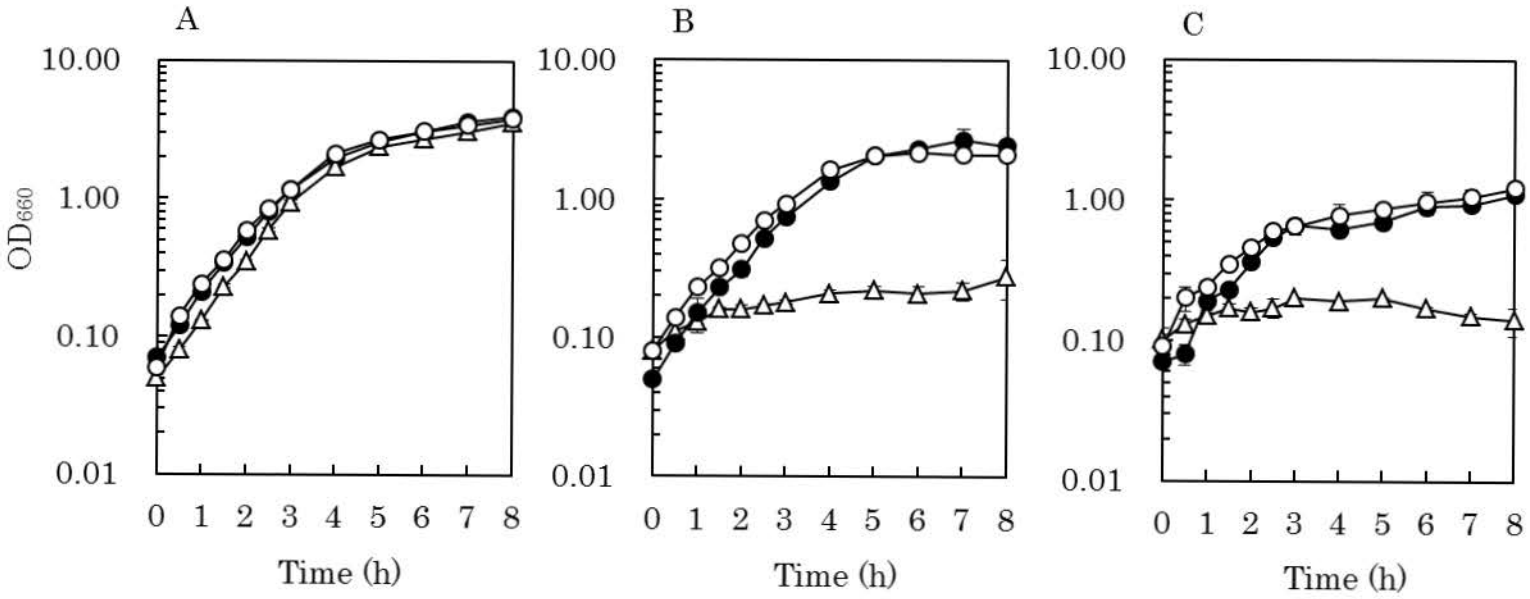

Fig. 2-4 Growth of *E. coli* JA300, JA300 *acrR*::IS *marR*, and CH7 in LBGMg liquid medium
Growth of strains in the absence of an organic solvent (A) and in the presence of *n*-hexane (B) and cyclohexane (C). A 100-µl culture of overnight-grown *E. coli* strain was inoculated to 10 ml of fresh LBGMg liquid medium overlaid with an organic solvent. This two-phase culture was incubated at 30° C. Growth was monitored by measuring turbidity (OD_{660}). Values indicate means of results and standard deviations of results from three independent experiments. Symbols: (△) JA300; (●) JA300 *acrR*::IS *marR*; (○) CH7.

2-4-5 AcrA, AcrB, and TolC levels in organic solvent tolerant mutants

Mutations in *marR* can increase the expression levels of AcrAB and TolC proteins, which are components of the AcrAB-TolC efflux pump (10). In addition, mutations in *acrR* can enhance the expression of AcrAB (23, 45). Levels of AcrA, AcrB, and TolC in JA300, CH7, JA300 *acrR*IS, JA300 *marR*, and JA300 *acrR*IS *marR* were investigated by immunoblotting analysis (Fig. 2-5). Both the AcrA and AcrB levels in JA300 *acrR*IS were about threefold higher than those in JA300. However, the TolC level in JA300 *acrR*IS was similar to that in JA300. The levels of AcrA, AcrB, and TolC in JA300 *marR* were about twice those in JA300. JA300 *acrR*IS *marR* exhibited higher expression levels of AcrA and AcrB compared to those of JA300 *acrR*IS and JA300 *marR*, but the TolC level in JA300 *acrR*IS *marR* was similar to that in JA300 *marR*. The levels of these three proteins in JA300 *acrR*IS *marR* were similar to those in strain CH7. These results suggested that the improved organic solvent tolerance in JA300 *acrR*IS *marR* and CH7 was a result of enhanced solvent-efflux activity by the overexpressed AcrAB-TolC pump.

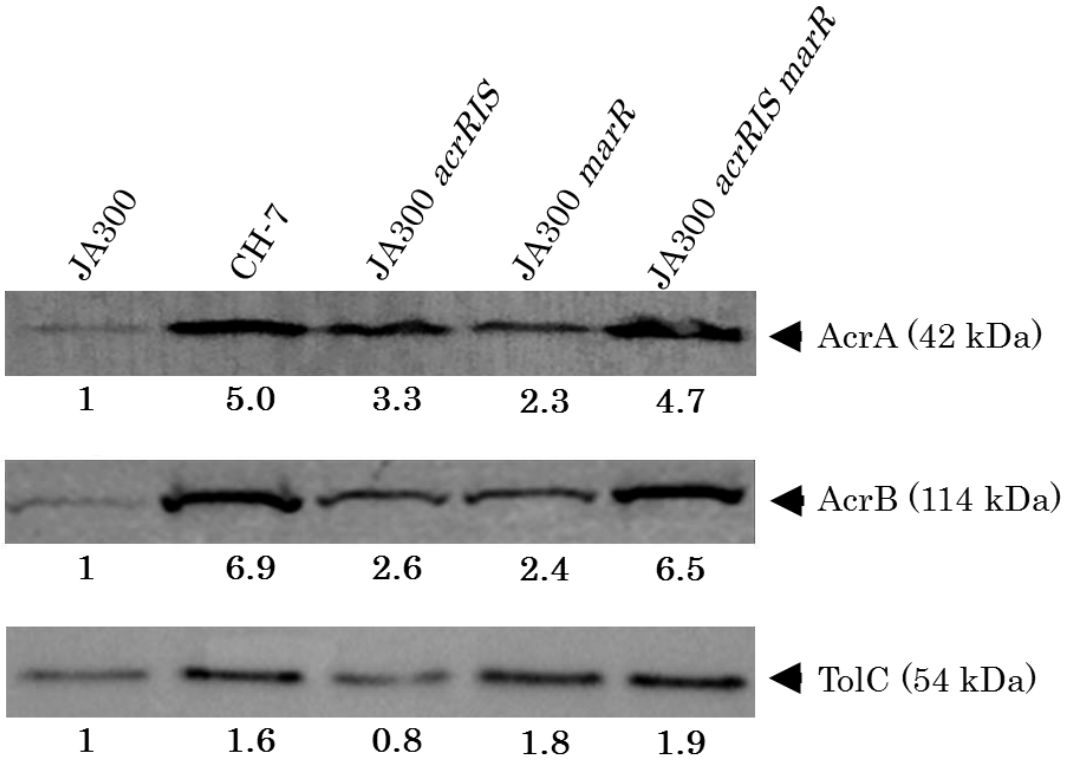

Fig. 2-5 Western blot analysis of AcrA, AcrB, and TolC expression
Total cell lysate proteins of JA300 (lane 1), CH7 (lane 2), JA300 *acrR*IS (lane 3), JA300 *marR* (lane 4), and JA300 *acrR*IS *marR* (lane 5) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with polyclonal anti-AcrA, AcrB, and TolC antibodies, respectively. The expression ratio compared to the AcrA level of JA300 is shown below each lane.

2-4-6 Organic solvent tolerance of *acrA*-disruptant

Since the *marR*109 mutation can influence the expression levels of many mar regulon genes, including *acrAB* and *tolC* (10), it was possible that mar regulon genes other than *acrAB* and *tolC* could be involved in the improved organic solvent tolerance in JA300 *acrR*::IS *marR*. To clarify the extent to which the AcrAB-TolC pump contributed to organic solvent tolerance in JA300 *acrR*::IS *marR*, a JA300 *acrR*::IS *marR*-based *acrA*-disruptant, JA300Δ*acrA* *acrR*::IS *marR*, was constructed and then the organic solvent tolerance of this *acrA*-disruptant was compared to that of JA300Δ*acrA* (Figure 2-6). The organic solvent tolerance of JA300Δ*acrA* was similar to that of the previously reported JA300-based *acrAB* disruptant (42). JA300 was tolerant to nonane ($\log P_{OW}$, 5.5) and octane ($\log P_{OW}$, 4.9). In contrast, JA300Δ*acrA* *acrR*::IS *marR* became sensitive to these solvents. The tolerance level of JA300Δ*acrA* *acrR*::IS *marR* was similar to that of JA300Δ*acrA*.

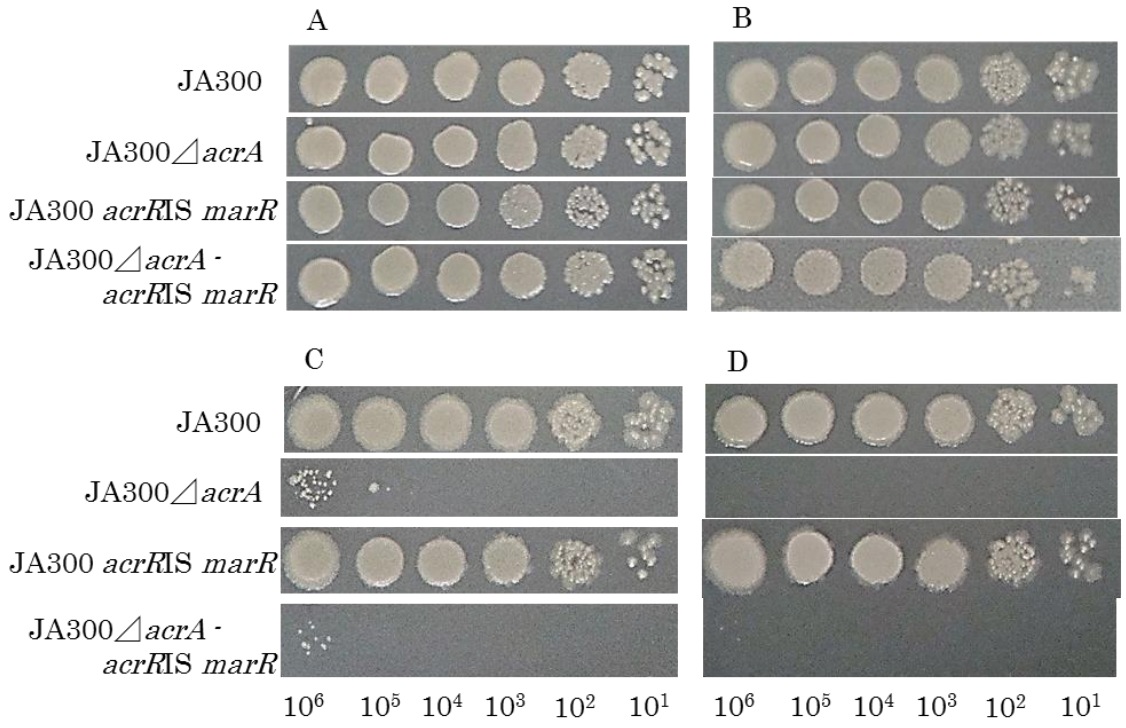

Fig. 2-6 Organic solvent tolerances of *acrA*-disruptant

Colony-forming efficiency of *acrA* disruptants of JA300 and JA300 *acrR*::IS *marR* on LBGMg agar medium in the absence of an organic solvent (A) and in the presence of decane (B), nonane (C), or octane (D). Each strain was spotted at a tenfold.

2-4-7 Accumulation of an organic solvent in *E. coli* incubated in a two-phase culture system

It has been reported that organic solvent tolerant *E. coli* strains in a two-phase culture system maintained low intracellular levels of organic solvents (12, 39, 42). The amounts of *n*-heptane ($\log P_{OW}$, 4.2), *n*-hexane ($\log P_{OW}$, 3.9), or cyclohexane ($\log P_{OW}$, 3.4) accumulated in *E. coli* cells were investigated (Table 2-3). *E. coli* cells of JA300, CH7, JA300 *acrR*IS, JA300 *marR*, or JA300 *acrR*IS *marR* were incubated for 30 min in the presence of organic solvents. The intracellular solvent levels of JA300 *acrR*IS and JA300 *marR* were similar to or slightly lower than those of the JA300 parent strain. On the other hand, the intracellular solvent levels of CH7 and JA300 *acrR*IS *marR* were remarkably lower than those of the JA300 parent strain. The amounts of *n*-heptane, *n*-hexane, and cyclohexane in CH7 were 6%, 20%, and 30% of those for JA300, respectively. On the other hand, the amounts of *n*-heptane, *n*-hexane, and cyclohexane in JA300*acrR*IS *marR* were 2%, 19%, and 22% of those for JA300, respectively. Thus, the intracellular amounts of organic solvents in JA300 *acrR*IS *marR* exhibited levels similar to those in CH7.

Table 2-3 Accumulation of organic solvents in *E. coli* cells in a two-phase system

Strain	Intracellular amount (mmol/mg of protein) of [a]:		
	n-Heptane	*n*-Hexane	Cyclohexane
JA300	0.060 ± 0.002	0.54 ± 0.01	1.3 ± 0.1
JA300 acrRIS	0.051 ± 0.037	ND[b]	1.2 ± 0.1
JA300 marR	0.049 ± 0.010	ND[b]	1.0 ± 0.1
JA300 acrRIS marR	0.0010 ± 0.0006	0.10 ± 0.01	0.29 ± 0.01
CH7	0.0033 ± 0.0002	0.11 ± 0.01	0.39 ± 0.01

[a] *E. coli* strains grown in LBGMg medium were exposed to organic solvents in the two-phase system and incubated for 30 min as described in Materials and Methods. Values indicate means of results and standard deviations of results from three independent experiments.
[b] ND, not determined.

2-4-8 Antibiotic tolerances of organic solvent tolerant mutants

Organic solvent tolerant mutants from *E. coli* were frequently tolerant to antibiotics including fluoroquinolone and hydrophobic antibiotics (19, 44). Thus, antibiotic tolerances of JA300, CH7, JA300 *acrR*IS, JA300 *marR* and JA300 *acrR*IS *marR* were investigated by assessing MICs of various antibiotics such as novobiocin, nalidixic acid, chloramphenicol, ofloxacin, and enoxacin (Table 2-4). JA300 *acrR*IS showed twofold increased MICs only against nalidixic acid and chloramphenicol as compared to JA300. This result suggested that increased expression of AcrAB lacking TolC in JA300 *acrR*IS seemed to confer low-level antibiotic tolerance on JA300 *acrR*IS. JA300 *marR* exhibited twofold increased MICs of novobiocin, nalidixic acid, chloramphenicol and ofloxacin as compared to JA300 but not show increased MICs of enoxacin. Both CH7 and JA300 *acrRIS marR* equally exhibited two- to four-fold increased MICs of all antibiotics as compared to JA300. Moreover, these two mutants also equally displayed higher antibiotic tolerance than JA300 *acrR*IS and JA300 *marR*. These results corresponded to levels of organic solvent tolerance and AcrAB-TolC efflux pump-expression in these strains.

Many fluoroquinolone-resistant clinical *E. coli* isolates displayed more than tenfold higher MICs of antibiotics such as nalidixic acid and chloramphenicol than JA300 *acrR*IS *marR* and CH7 (19, 44). These clinical isolates carried mutations causing not only overexpression of AcrAB-TolC efflux pump but also alternations in drug targets (e.g., DNA gyrase or topoisomerase IV). It was interesting that these clinical isolates showing

higher antibiotics-resistance than JA300 *acrR*IS *marR* and CH7 were more sensitive to organic solvents than JA300 *acrR*IS *marR* and CH7 (44).

.

Table 2-4 Antibiotic tolerance of organic solvent-tolerant *E. coli* mutants

Strain	MIC (mg/ml) for:				
	NOV	NAL	CHL	OFL	ENO
JA300	200	3.1	3.1	0.078	0.31
JA300 *acrR*::S	200	6.3	6.3	0.078	0.31
JA300 *marR*	400	6.3	6.3	0.16	0.31
JA300 *acrR*::S *marR*	800	12.5	12.5	0.31	0.63
CH7	800	12.5	12.5	0.31	0.63

Abbreviations: NOV, Novobiocin; NAL, Nalidixic acid; CHL, Chloramphenicol; OFL, Ofloxacin; ENO, Enoxacin.

2-5 Conclusions

In the present study, we isolated cyclohexane-tolerant mutants from cyclohexane-sensitive *E. coli* K-12 strain JA300 and investigated whether or not these mutants carried mutations in regulatory genes *marR*, *soxR*, and *acrR*. Most of the mutants carried mutations in *marR*. Three of the seven mutations found in *marR* caused amino acid substitutions in MarR at the amino acid positions of L78, R94, and G116, and four of the seven mutations led to a translation termination codon at the position of E109 (*marR*109 mutation). Three mutants isolated in this study carried different mutations in *acrR*. Two mutations in *acrR* caused amino acid substitutions in AcrR at the amino acid positions of M1 and A41. Many IS elements have been shown to activate or inactivate the expression of neighboring genes. In strain CH7, the IS*5* inserted within *acrR* seemed to activate the expression of *acrAB* through the disruption of transcriptional repression by AcrR. However, there was no mutation in *soxR* in cyclohexane-tolerant mutants. Two mutants (CH1 and CH7) carried mutations in both *marR* and *acrR*. These mutants exhibited higher organic solvent tolerances than other isolates (Fig. 2-1). In particular, strain CH7 containing *marR*109 and *acrR*::IS showed the highest organic solvent tolerance among all isolates.

It was possible that unidentified mutations other than *marR109* and *acrR*::IS5 might influence organic solvent tolerance in strain CH7. To clarify the effect of the *marR109* and/or *acrR*::IS5 mutations on organic solvent tolerance in *E. coli*, JA300 *acrR*IS, JA300 *marR*, and JA300 *acrR*IS *marR* were constructed. A comparison of the tolerances in these mutants and in

strain CH7 revealed that the improved organic solvent tolerance in strain CH7 was caused by a synergistic effect of the double mutations of *marR* and *acrR*.

The AcrAB-TolC efflux pump is involved in organic solvent tolerance in *E. coli* (42). The order of organic solvent tolerances of JA300 *acrR*IS, JA300 *marR*, and JA300 *acrR*IS *marR* was comparable to the order of the expression levels of AcrAB and TolC (Fig. 2-3 and 2-5). The expression levels of AcrA and AcrB proteins in JA300 *acrR*IS were similar to, or slightly higher than, the levels in JA300 *marR*. However, the extent of improvement in organic solvent tolerance in JA300 *acrR*IS was lower than that in JA300 *marR* because the disruption of *acrR* did not influence the expression level of TolC. JA300 *acrR*IS *marR* and CH7 equally enhanced the expression levels of AcrAB and TolC compared to JA300 *acrR*IS and JA300 *marR*. In addition, the intracellular solvent levels of JA300 *acrR*IS *marR* and CH7 were similarly kept lower than those of JA300 *acrR*IS, JA300 *marR*, and JA300. These results suggested that the improved organic solvent tolerance in JA300 *acrR*IS *marR* and CH7 was a result of enhanced solvent-efflux activity by the overexpressed AcrAB-TolC pump. To clarify the contribution of the AcrAB-TolC pump to organic solvent tolerance in JA300 *acrR*IS *marR*, an *acrA*-disruptant (JA300Δ*acrA* *acrR*IS *marR*) was constructed and its organic solvent tolerance was compared to that of JA300Δ*acrA* (Fig. 2-6). JA300Δ*acrA* *acrR*IS *marR* became as sensitive to organic solvents as JA300Δ*acrA*. This result indicated that the AcrAB-TolC pump is essential for JA300 *acrR*IS *marR* to acquire high-level organic solvent tolerance. In

addition, it suggested that the mar regulon genes other than *acrAB* and *tolC* are barely involved in organic solvent tolerance in JA300 *acrR*IS *marR*.

Organic solvent tolerant *E. coli* mutants are known to exhibit resistance to a variety of antibiotics (6, 8). Therefore, the antibiotic tolerances of JA300 *acrR*IS, JA300 *marR*, JA300 *acrR*IS *marR* and strain CH7 were examined (Table 2-4). JA300 *acrR*IS showed the increased tolerance only against nalidixic acid and chloramphenicol. JA300 *marR* exhibited the tolerance against a wider range of antibiotics than JA300 *acrR*IS. Moreover, JA300 *acrR*IS *marR* and strain CH7 equally displayed higher antibiotic tolerance than JA300 *acrR*IS and JA300 *marR*. These results corresponded to levels of organic solvent tolerance and AcrAB-TolC efflux pump-expression in these strains. It has been reported that many fluoroquinolone-resistant clinical *E. coli* isolates displayed more than tenfold higher MICs of antibiotics such as nalidixic acid and chloramphenicol than JA300 *acrR*IS *marR* and CH7 (19, 44). However, these clinical isolates are more sensitive to organic solvents than JA300 *acrR*IS *marR* and CH7, although these show higher antibiotics resistance than JA300 *acrR*IS *marR* and CH7 (44). These results suggested that these clinical isolates carried mutations causing not only overexpression of AcrAB-TolC efflux pump but also alternations in drug targets.

In this study, it was clarified that only two mutations in regulatory genes in *acrR* and *marR* confer high-level organic solvent tolerance on *E. coli*. Owing to the wealth of genetic and metabolic knowledge associated with *E. coli*, organic solvent tolerant *E. coli* can be a convenient and efficient catalyst when it is used as a host expressing enzymes that are useful for producing

valuable chemicals in two-phase systems employing organic solvents. The present findings are expected to provide valuable knowledge for increasing organic solvent tolerance levels in *E. coli* to improve the usability of whole-cell biocatalysts in two-phase systems.

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Chapter 3

Improvement of organic solvent tolerance by disruption of the *lon* gene in *Escherichia coli*

3-1 Abstract

The Lon is an ATP-dependent protease belonging to the AAA+ (ATPases associated with a variety of cellular activities) superfamily of enzymes. The Lon plays an important role in regulating many biological processes in bacteria. In the present study, we investigated the organic solvent tolerance of a ⊿*lon* mutant of *Escherichia coli* K-12 and found that the mutant showed significantly higher organic solvent tolerance than the parent strain. ⊿*lon* mutants are known to overproduce capsular polysaccharide and concomitantly form mucoid colonies. Thus, it was possible that this increase in capsular polysaccharide production might be involved in the organic solvent tolerance in *E. coli*. However, this study showed that a ⊿*lon* ⊿*wcaJ* double-gene mutant displaying a nonmucoid phenotype was as tolerant to organic solvents as the ⊿*lon* mutant. This result indicated that capsular polysaccharide is not involved in organic solvent tolerance. On the other hand, the Lon protease is known to cause rapid turnover of MarA and SoxS, which can enhance the expression level of the AcrAB-TolC efflux pump. We found that the ⊿*lon* mutant showed a higher expression level of AcrB than the parent strain. In addition, the ⊿*lon* ⊿*acrB* double-gene mutant showed a significant decrease in organic solvent tolerance. Thus, it was indicated that organic solvent tolerance in the ⊿*lon* mutant depends on the AcrAB-TolC pump but not capsular polysaccharide. As described in the

previous chapter, we constructed *E. coli* strain JA300 *acrR*::IS *marR*. This *E. coli* mutant overexpresses the AcrAB-TolC pump and exhibits high-level solvent tolerance. In an attempt to further improve the solvent tolerance of JA300 *acrR*::IS *marR*, a *lon* gene disruptant of this strain was constructed. However, the resulting mutant JA300 *acrR*::IS *marR* ⊿*lon* showed lower solvent tolerance than JA300 *acrR*::IS *marR*. In addition, we examined antibiotic susceptibilities of the⊿*lon* mutant. The ⊿*lon* mutant did not exhibit a remarkable multidrug resistance-phenotype.

3-2 Introduction

Many attempts have been made to produce valuable compounds through the bioconversion of hydrophobic and/or toxic organic compounds in two-phase systems consisting of a hydrophobic organic solvent and an aqueous medium (13, 14, 29, 30). In addition, increasing attention has been paid to microbial production of hydrophobic biofuels such as long-chain alcohols and fatty acid-based fuels from renewable biomass resources (7, 17, 26). In a two-phase culture system containing a large amount of a hydrophobic organic solvent, the extent of bacterial growth inhibition is inversely correlated with the log P_{OW} of the solvent (15). Organic solvents with a log P_{OW} of 2 to 5 intercalate into the cell membrane and disrupt the integrity of the membrane, thereby affecting several vital functions, such as membrane transport and energy generation (4, 33).

Various mechanisms related to microbial tolerance and response to solvents have been proposed for *Pseudomonas* species and *E. coli* strains (3,

11, 13, 21-24, 28, 31, 32, 36, 42). In *E. coli*, the AcrAB-TolC efflux pump belonging to the resistance/nodulation/cell division (RND) family has been shown to provide intrinsic tolerance to organic solvents (38). *acrAB* and *tolC* are *marA/soxS/rob* regulon genes (9). The *acrAB* and *tolC* expression is activated by transcriptional activators such as MarA and SoxS proteins (Fig. 1-4) (1). Transcriptions of the *marA* and *soxS* genes are repressed by MarR and SoxR, respectively. In addition, *acrAB* expression is modulated locally by the repressor AcrR (18). In chapter 2, it was described that an *E. coli* strain could acquire high-level organic solvent tolerance via mutations in only the two regulatory genes *marR* and *acrR* (42).

The Lon protease plays an essential role in protein quality control by destroying unfolded proteins (Fig. 3-1) (37). The *E. coli* Lon protease has been shown to be involved in a number of biological processes, such as SOS response, capsule synthesis, DNA methylation, motility, defense against chemicals, methionine biosynthesis, acid tolerance, and nutrient stress (37, 41). The Lon protease causes a rapid turnover of MarA and SoxS (12). Therefore, MarA and SoxS are unstable in the presence of Lon protease. *lon* mutants increase the expression level of the AcrAB-TolC pump (20). In addition, *lon* mutants enhance the production of a capsular polysaccharide, colanic acid (Fig. 3-2), and this leads to a mucoid phenotype (35). The enhanced polysaccharide biosynthesis has been thought to cause increased antibiotic resistance via decreased permeability (19). In addition, extracellular polysaccharide is suggested to play an important role in organic solvent tolerance in several bacteria (16, 25, 40, 43). Thus, we expected that

overproduction of capsule polysaccharide would contribute to organic solvent tolerance in *E. coli*. The colanic acid biosynthesis requires 19 genes located on the same cluster, denoted *wca* and formerly called *cps* (34). WcaJ is predicted to initiate the synthesis of colanic acid by transferring α-D-glucose-1-phosphate to undecaprenyl phosphate. WcaJ is required for capsule polysaccharide synthesis, and the ⊿*wcaJ* mutant forms nonmucoid colonies (Fig. 3-3).

In this study, the organic solvent tolerance of an *E. coli* ⊿*lon* mutant was examined and it was found that the mutant showed higher organic solvent tolerance than the parent strain. In addition, this study clarified the contribution of the capsular polysaccharide and the AcrAB-TolC pump to organic solvent tolerance in the ⊿*lon* mutant.

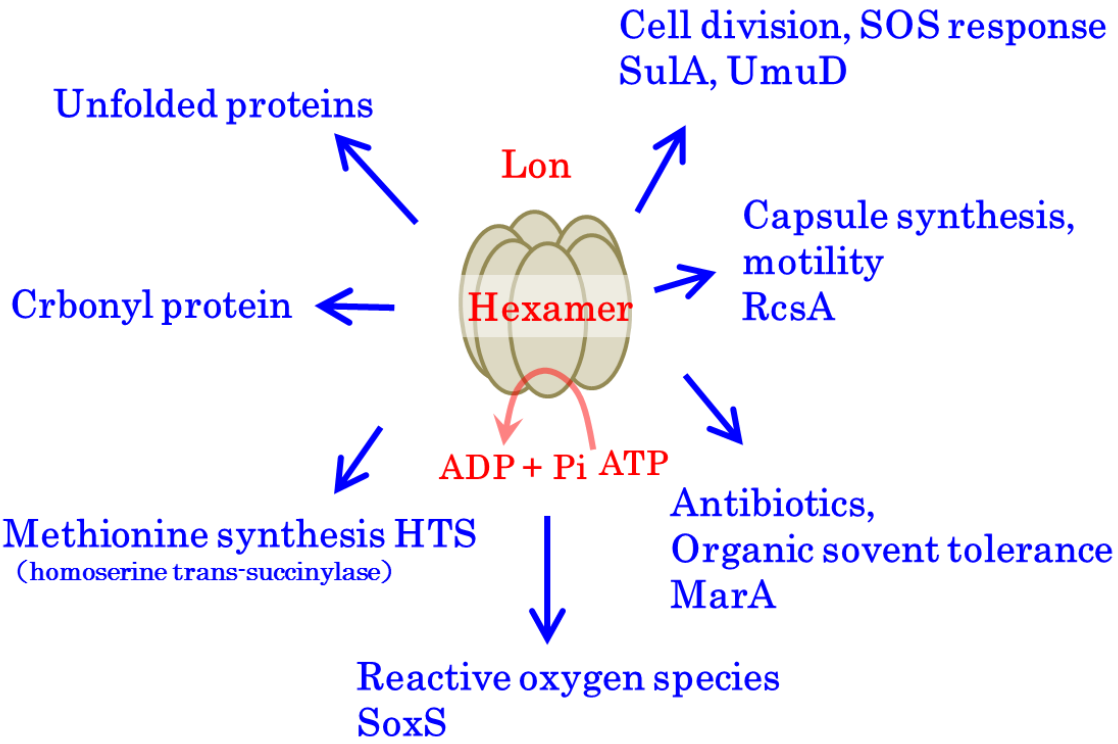

Fig. 3-1 Lon protease

The Lon is hexamer and has ATP binding motif. The protease plays an essential role in protein quality control by destroying unfolded proteins. The *E. coli* Lon protease has been shown to be involved in a number of biological processes.

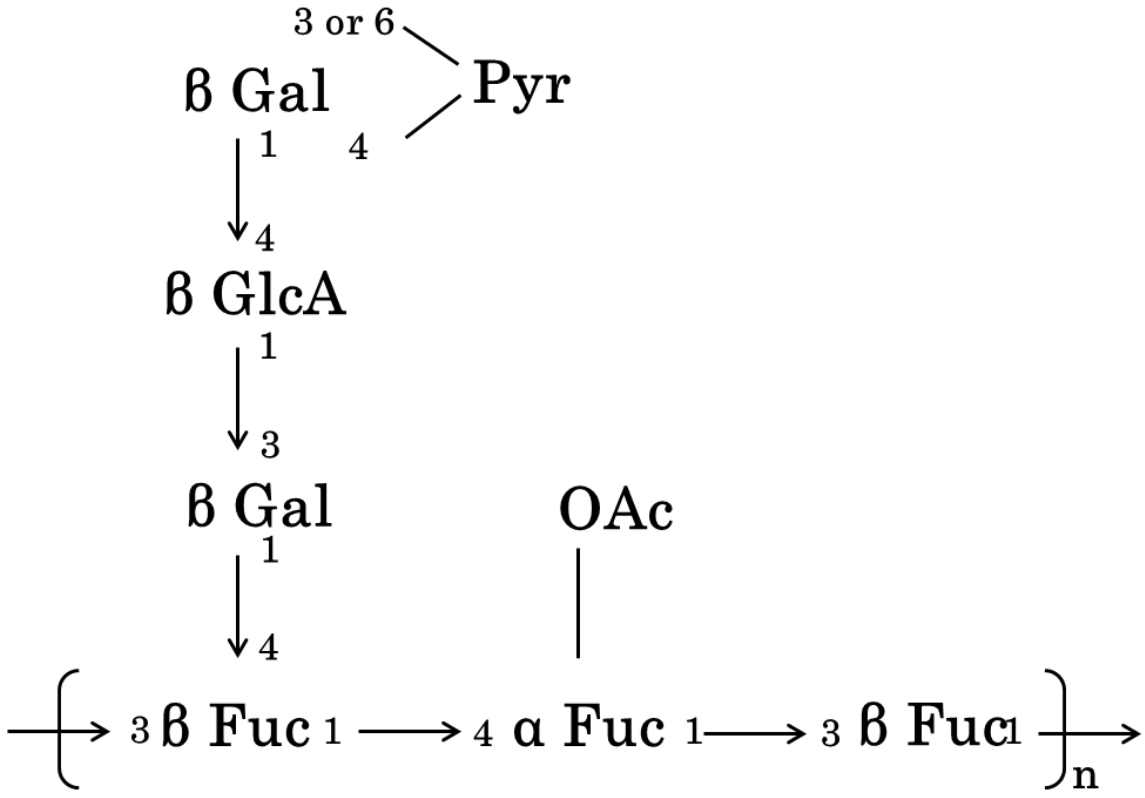

Fig. 3-2 Chemical structure of the colanic acid monomer
Fuc:L-fucose, Gal:D-galactose, GlcA:D-glucuronic acid, Glc:D-glucose, Oac:O-acetyl, Pyr:pyruvate.

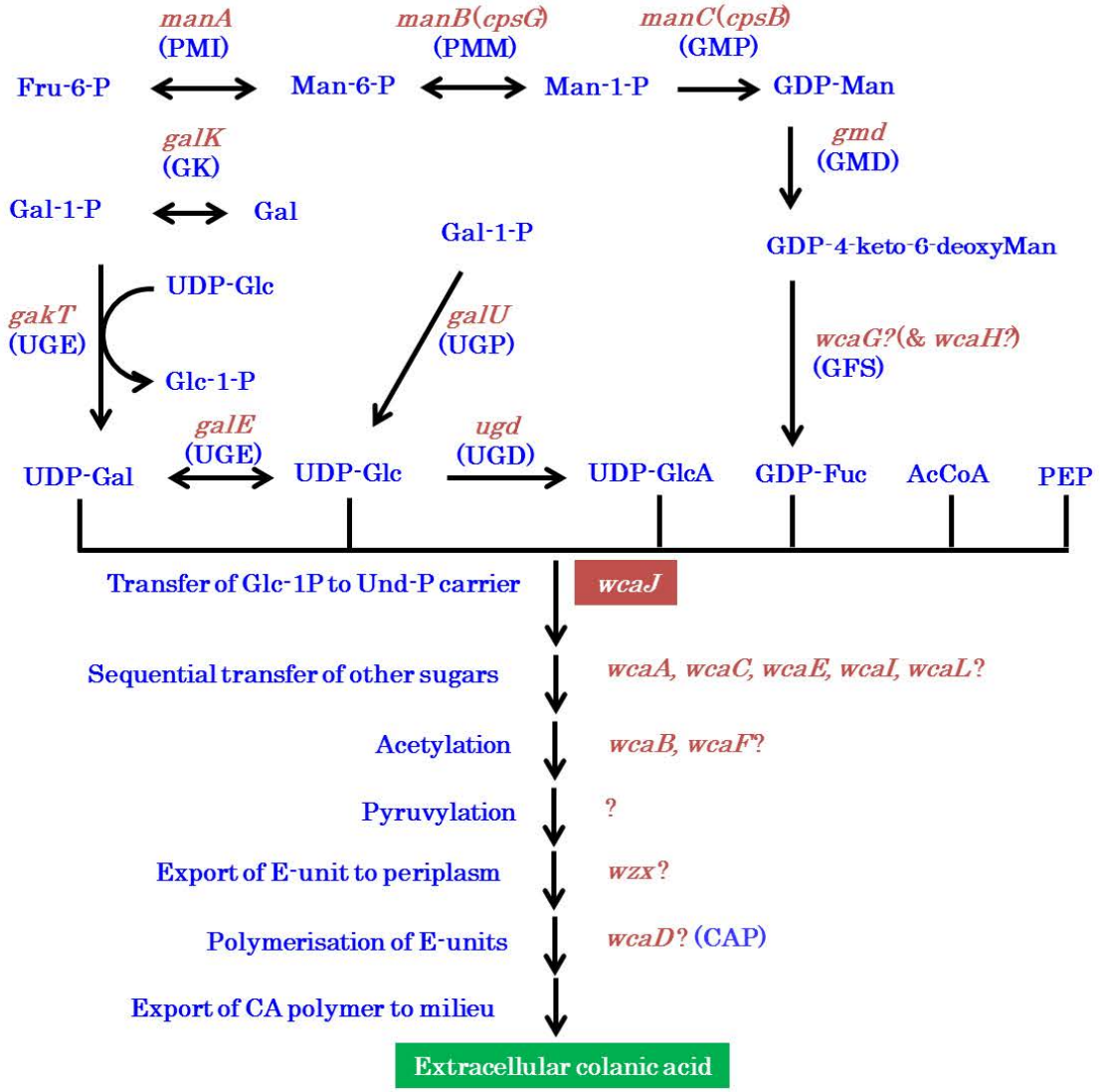

Fig. 3-6 Organic solvent tolerances of strain JA300 *acrR*IS *marR* and JA300 *acrR*IS *marR*△*lon* mutant
Colony-forming efficiency of JA300, JA300 *acrR*IS *marR* and JA300 *acrR*IS *marR* △*lon* on LBGMg agar medium in the absence of an organic solvent (A) and in the presence of *n*-hexane (B), and cyclohexane (C).

3-3 Materials and methods

3-3-1 Media, culture conditions and materials

The organisms were grown aerobically at 30°C in LBG medium consisting of 1% Bacto Tryptone (Difco Laboratories, Detroit, MI), 0.5% Bacto Yeast Extract (Difco), 1% NaCl, and 0.1% glucose. The same medium supplemented with 10 mM $MgSO_4$ (LBGMg medium) (27) was also used. The LBGMg medium was solidified with 1.5% (wt/vol) agar. Ampicillin (50 μg/ml) or kanamycin (50 μg/ml) was added to the medium when necessary. The organic solvents used were of the highest quality available (Wako Pure Chemical Industries, Osaka, Japan). The hydrophobic solvents used and their $\log P_{ow}$ values were as follows: *n*-nonane ($\log P_{ow}$, 5.5), *n*-hexane ($\log P_{ow}$, 3.9), cyclohexane ($\log P_{ow}$, 3.4) and *p*-xylene ($\log P_{ow}$, 3.1).

3-3-2 Bacterial strains and plasmids

The *E. coli* K-12 derivatives used to evaluate solvent tolerance are summarized in Table 3-1. Strain BW25113 and its single-gene knockout mutants were supplied by the National Bio-Resource Project (NIG, Mishima, Japan): *E. coli* (8). The plasmid pCP20 was also obtained from NIG, Japan.

Table 3-1 Bacterial strains used

E. coli strain	JW ID[a]	Genotype	Reference
BW25113		*lacI*^{q} *rrnB*$_{T14}$ *lacZ*$_{WJ16}$ *hsdR*514 *araBAD*$_{AH33}$ *rhaBAD*$_{LD78}$	(34)
BW25113 ⊿*lon*	JW0429	Same as BW25113, but with *lon*::Km^{R}	(34)
BW25113 ⊿*acrB*	JW0451	Same as BW25113, but with *acrB*::Km^{R}	(34)
BW25113 ⊿*wcaJ*	JW2032	Same as BW25113, but with *wcaJ*::Km^{R}	(34)
BW25113 ⊿*lon*⊿*acrB*		Same as BW25113, but with ⊿*acrB* and *lon*::Km^{R}	This study
BW25113 ⊿*lon*⊿*wcaJ*		Same as BW25113, but with ⊿*wcaJ* and *lon*::Km^{R}	This study
JA300		F^{-} *thr leuB6 trpC1117 thi rpsL20 hsdS*	(43)
JA300 *acrR*::IS *marR*		Same as JA300, but with *acrR*::IS5 and *marR*109	(36)
JA300 *acrR*::IS *marR* ⊿*lon*		Same as JA300, but with *lon*::Km^{R}, *acrR*::IS5 and *marR*109	This study

[a]JW ID of the Keio collection by the National Bio-Resource Project (NIG, Mishima, Japan): *E. coli* (34).

3-3-3 Disruption of *lon*, *acrB*, and *wcaJ* in *E. coli* strains

The plasmid pCP20 shows temperature-sensitive replication and thermal induction of FLP synthesis (10). The Km^R cassettes in BW25113⊿*acrB* and BW25113 ⊿ *wcaJ* were eliminated with pCP20. These strains were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which several were colony-purified at 43°C and then tested for loss of kanamycin-resistance. Elimination of the Km^R cassette was confirmed by PCR analysis using chromosomal DNA. The combination of primers for BW25113⊿*acrB* was acrB-S and acrB-AS, and that for BW25113 ⊿*wcaJ* was cpsGwcaJ-S and wcaJ-AS (Table 3-2). The sizes of the amplified products from BW25113⊿*acrB* and BW25113⊿*wcaJ* were estimated to be 1.7 kb and 3.1 kb, respectively. Consistent with the expected values, the sizes of the amplified products from the Km^R cassette-eliminated strains BW25113⊿*acrB* and BW25113⊿*wcaJ* were 0.2 kb and 1.7 kb, respectively. BW25113⊿*lon*⊿*acrB* and BW25113⊿*lon*⊿*wcaJ* were constructed from the Km^R cassette-eliminated mutants BW25113⊿*acrB* and BW25113⊿*wcaJ*, respectively, by P1 transduction of kanamycin-resistance with BW25113⊿ *lon* as the donor. Disruption of the *lon* gene was confirmed by PCR analysis using chromosomal DNA prepared from BW25113⊿*lon*⊿*acrB* or BW25113 ⊿*lon*⊿*wcaJ* as the template with the combination of primers lon-S and lon2-AS (Table 3-2). JA300⊿*lon* and JA300 *acrR*IS *marR* ⊿*lon* mutants were also constructed from JA300 and JA300 *acrR*IS *marR*, respectively, by P1 transduction of kanamycin-resistance with BW25113⊿*lon* as the donor. Disruption of the *lon* gene was confirmed by PCR analysis with primers

lon-S and lon2-AS. Corresponding to the expected values, the sizes of the amplified products from both BW25113 and JA300 were 2.4 kb and those of the products from the Km^R cassette-eliminated strains (BW25113⊿*lon*⊿*acrB*, BW25113⊿*lon*⊿*wcaJ*, JA300⊿*lon*, and JA300 *acrR*IS *marR* ⊿*lon*) were all approximately 1.5 kb.

3-3-4 Measurement of organic solvent tolerance of *E. coli*

Cultures of *E. coli* strains in LBGMg medium after 16 h of incubation (optical density at 660 nm [OD_{660}], 4 to 5) were diluted with 0.8% saline by serial 10-fold dilutions. Each suspension was plated on LBGMg agar. The agar surface was overlaid with a 3-mm-thick layer of an organic solvent. The approximate frequency at which the cells formed colonies on the agar was estimated after 48 h of incubation at 25°C.

Table 3-2 Primers used in this study.

Primer	Sequence (5′ to 3′)	Positions
acrB-S	CGCTGATAATAACCAGCAAG	61-80 bp upstream of the initiation codon of *acrB*
acrB-AS	GTTGGTGGTTCAATTACTCC	64-83 bp downstream of the stop codon of *acrB*
cpsGwcaJ-S	AGGCAATGAATGCGAAACCG	75-94 bp upstream of the initiation codon of *cpsG*
wcaJ-AS	TGATGATCACCGTGGCAATC	46-65bp downstream of the stop codon of wcaJ
lon-S	CATCTGATTACCTGGCGGAA	22-41bp upstream of the initiation codon of *lon*
lon2-AS	CCTGCCAGCCCTGTTTTTAT	23-42 bp downstream of the stop codon of *lon*

3-3-5 Antibodies against AcrB

Polyclonal antibody against AcrB was raised against synthetic peptides corresponding to regions of AcrB. This antibodies contained an N-terminal cysteine (C + in the peptides shown below) for the conjugation of keyhole limpet hemocyanin. The peptide sequences were as follows: a synthetic peptide for anti-AcrB antibodies, C + KNEDIEHSHTVDHH (corresponding to residues 1036 to 1049). The conjugated peptide was injected into rabbits, and polyclonal antibodies were purified from serum using a Melon gel immunoglobulin G spin purification kit (Thermo Fisher Scientific, Rockford, IL, USA). This antibodies was used to detect AcrB in the immunoblotting analyses of this study.

3-3-6 Immunoblotting analysis

E. coli cells grown in LBGMg medium to an OD_{660} of about 0.6 were harvested by centrifugation (4,400 × *g* for 10 min at 4°C), suspended in 10 mM Tris-HCl buffer (pH 8.0), and then sonicated on ice. Ten micrograms of total cell lysate protein in the supernatant was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12.5% separating gel (39). The gel was then electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA) by the application of 50 V for 30 min in Tris-glycine-methanol buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol [pH 8.3]). The membrane was blocked overnight at room temperature in Tris-buffered saline (TBS; 0.15% NaCl, 10 mM Tris-HCl, pH 7.4) containing 3% gelatin,

washed twice in wash buffer (0.05% Tween 20 in TBS), and hybridized at room temperature with anti-AcrB antibody. The membrane was then probed for 1 h with goat anti-rabbit horseradish peroxidase (Bio-Rad, Hercules, CA, USA). The bands were visualized by use of an alkaline phosphatase color development kit (Bio-Rad). The AcrB expression levels were quantified by gel analysis software UN-SCAN-IT (Silk Scientific, Orem, UT, USA).

3-3-7 Protein content

The protein concentration was determined using the method of Bradford (39) and bovine serum albumin as the standard.

3-3-8 Antibiotic susceptibility

The minimum inhibitory concentrations (MICs) of various antibiotics were determined by a sequential dilution method (6). LBG medium liquid cultures containing different concentrations of antibiotic and freshly grown 10^3 cells of the tested *E. coli* strain were incubated at 37°C for 16 h. The lowest concentration of antibiotic which completely inhibited growth was defined as the MIC (6).

3-4 Results and discussion

3-4-1 Organic solvent tolerances of strain BW25113 and its gene-knockout mutants

The organic solvent tolerances of the BW25113-based ⊿*lon*, ⊿*acrB*, ⊿*wcaJ*, ⊿*lon*⊿*acrB* and ⊿*lon*⊿*wcaJ* mutants were investigated by

measuring the colony-forming efficiency on an agar plate overlaid with pure decane, pure *n*-nonane, pure *n*-octane, pure *n*-heptane, pure *n*-hexane, a mixture of *n*-hexane and cyclohexane (9:1 vol/vol mixture), and pure cyclohexane (Fig. 3-4). All strains formed colonies in all spots on the plates without any solvent and with decane, respectively. The *lon* gene disruptants, BW25113⊿*lon* and BW25113⊿*lon*⊿*acrB*, formed mucoid colonies. The capsular polysaccharide synthesis leading to the mucoid colonies formation was partially suppressed by the addition of organic solvents. BW25113⊿*lon* exhibited 10^2- and 10^3-fold higher colony-forming efficiencies than the parent strain BW25113 in the presence of *n*-hexane and the solvent mixture, respectively. Extracellular polysaccharide has been reported to be involved in organic solvent tolerance in several bacteria as follows. *Rhodococcus* sp. 33 efficiently degrades benzene and produces a large quantity of extracellular polysaccharide which plays an important role in the benzene tolerance (40). *Rhodococcus rhodochrous* (a mucoidal strain) producing extracellular polysaccharide is resistant to *n*-hexadecane, while its nonmucoidal derivatives are sensitive to this solvent (16). *Staphylococcus* sp. ZZ1 produced an unusual extracellular capsule in response to toluene, suggesting that the hydrophilic carbohydrate capsule repels organic solvents and prevents them from reaching the cell membrane (43). *Acinetobacter venetianus* Rag-1 increased production of the capsular polymer emulsan in the presence of organic solvents (25). These findings in capsule polysaccharide production in bacteria are indicative of common survival strategies to withstand organic solvent stress. Thus, it was expected that the

overproduction of capsule polysaccharide might improve organic solvent tolerance also in *E. coli*.

To clarify the involvement of the AcrAB-TolC efflux pump and/or capsular polysaccharide in the organic solvent tolerance in BW25113⊿*lon*, the organic solvent tolerances of BW25113⊿*lon*⊿*acrB* and BW25113⊿*lon*⊿*wcaJ* together with BW25113⊿*acrB* and BW25113⊿*wcaJ* were examined. Unlike BW25113⊿*lon*, BW25113⊿*lon*⊿*wcaJ* formed nonmucoidal colonies. The organic solvent tolerances of BW25113⊿*wcaJ* and BW25113⊿*lon*⊿*wcaJ* were similar to those of the BW25113 and BW25113⊿*lon* strains, respectively. Thus, the increased capsular polysaccharide synthesis was not involved in the organic solvent tolerance in the ⊿*lon* mutant. On the other hand, both BW25113⊿*lon*⊿*acrB* and BW25113⊿*acrB* were highly sensitive to organic solvents. Therefore, the efflux pump was shown to significantly contribute to organic solvent tolerance in the *lon* mutant.

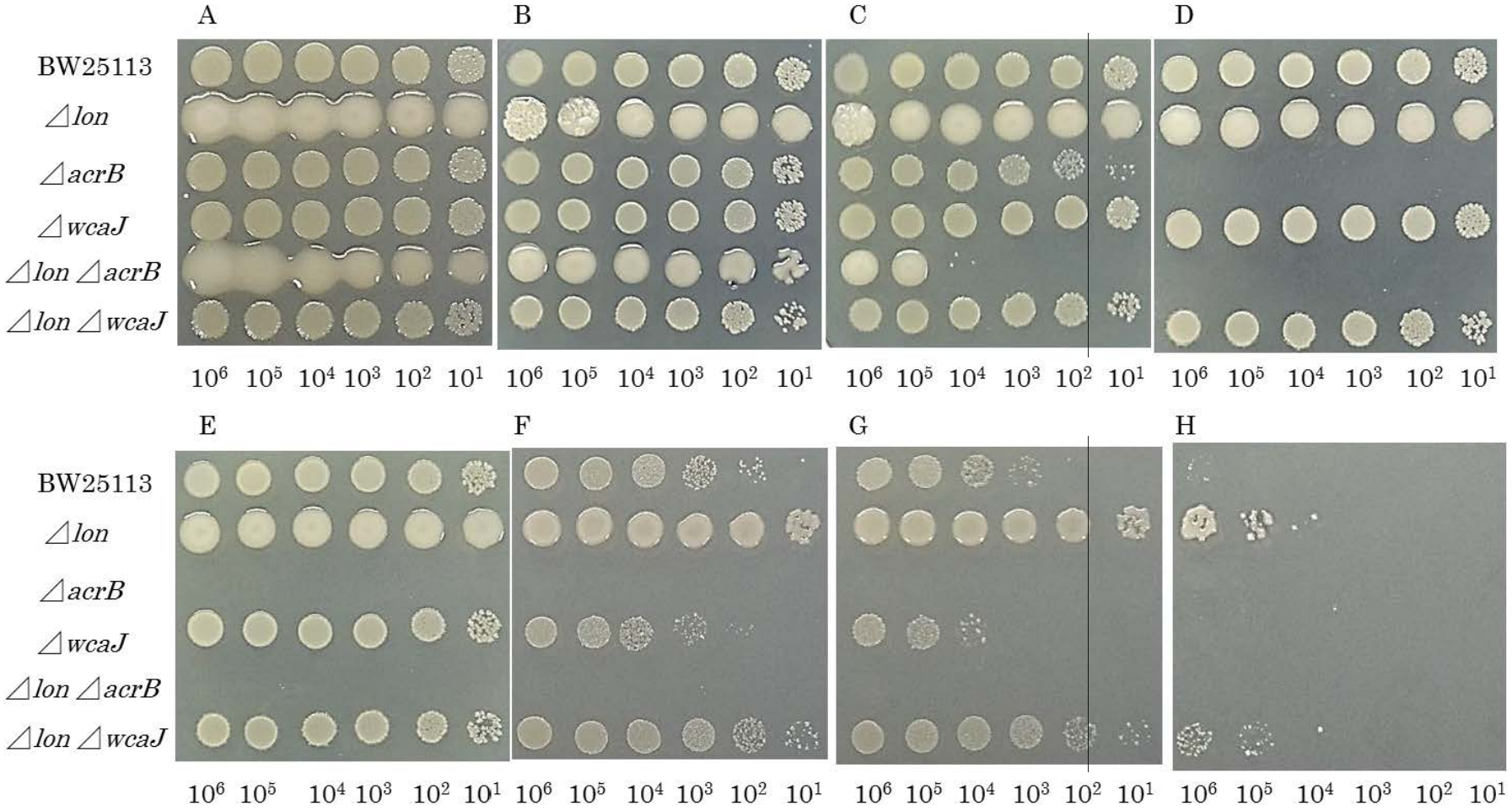

Fig. 3-4 The organic solvent tolerances of the BW25113-based D*lon*, D*acrB*, D*wcaJ*, D*lon*D*acrB* and D*lon*D*wcaJ* mutants
The colony-forming efficiency on an agar medium in the absence of an organic solvent (A) and in the presence of pure decane (B), pure *n*-nonane (C), pure *n*-octane (D), pure *n*-heptane (E), pure *n*-hexane (F), a mixture of *n*-hexane and cyclohexane (9:1 vol/vol mixture) (G), and pure cyclohexane (H).

3-4-2 Growth of the ⊿*lon* mutant in liquid medium in the presence of organic solvents

The cell growth of BW25113 and BW25113⊿*lon* in the LBGMg liquid medium in the presence of a hydrophobic solvent mixture of *n*-hexane and cyclohexane (9:1 vol/vol) and a hydrophilic solvent (ethanol or *n*-butanol) was examined by measuring turbidity (Fig. 3-5). No significant difference in growth was found among these strains in the absence of organic solvents (Fig. 3-5 A). As shown Fig. 3-5 B, the growth of BW25113 was highly suppressed by the addition of the hydrophobic solvent. In contrast, BW25113⊿*lon* was able to grow in the presence of the hydrophobic solvent, although the growth rate and yield of the mutant were partially reduced compared to those in the absence of solvent. On the other hand, the growth of BW25113⊿*lon* was somewhat suppressed compared to the growth of BW25113 in the presence of ethanol and *n*-butanol (Fig. 3-5 C and D). Since hydrophilic solvents are thought to have higher affinity to hydrophilic capsular polysaccharide than hydrophobic solvents, the *lon* mutant might be sensitive to hydrophilic solvents rather than hydrophobic solvents. On the other hand, the AcrAB-TolC pump has been reported to be not involved in the tolerance against short-chain alcohols (2).

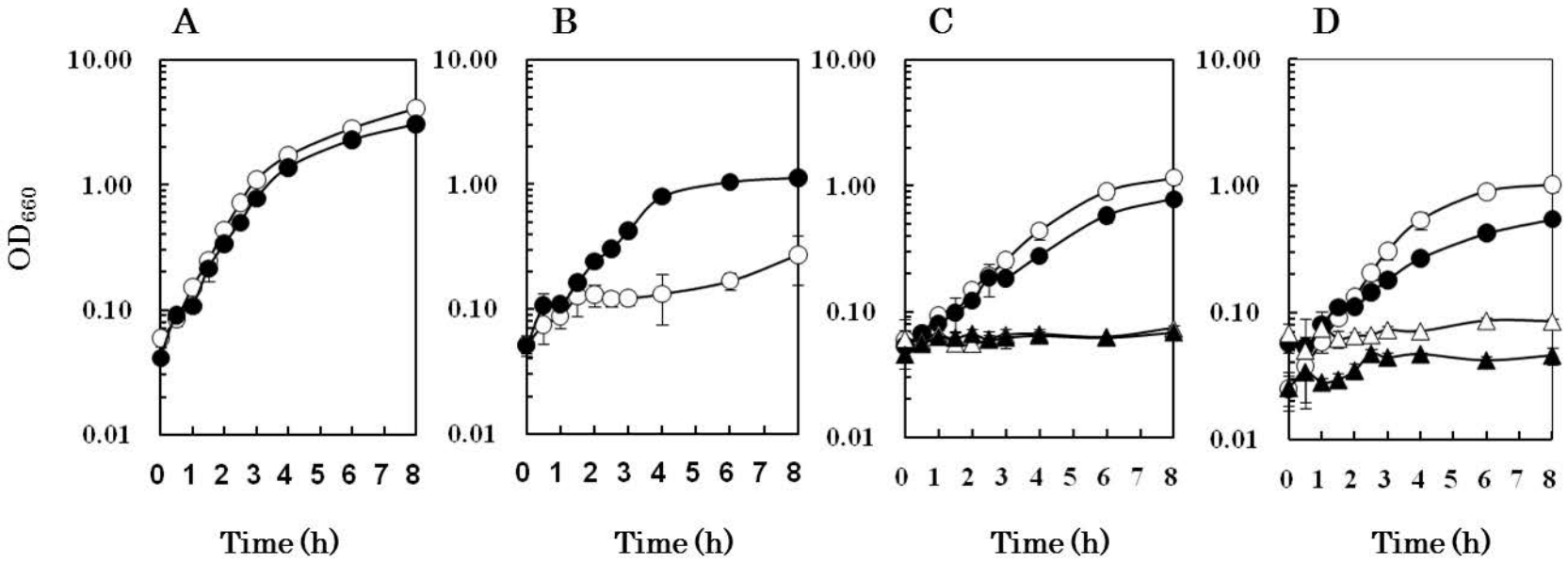

Fig. 3-5 Growth of the ⊿*lon* mutant in liquid medium in the presence of organic solvents
Growth of *E. coli* BW25113 and BW25113⊿*lon* in LBGMg liquid medium in the absence of an organic solvent (A) and in the presence of a 10% (vol/vol) *n*-hexane and cyclohexane mixture (9:1 vol/vol) (B), 5% or 10% (vol/vol) ethanol (C) and 1% or 2% (vol/vol) *n*-butanol (D). A 100-ml culture of overnight-grown *E. coli* strain was inoculated into 10 ml of fresh LBGMg liquid medium containing an organic solvent. This two-phase culture was incubated at 30° C. Growth was monitored by measuring turbidity (OD_{660}). Values indicate the means of results and standard deviations of results from three independent experiments. Open and filled symbols represent BW25113 and BW25113⊿*lon*, respectively. (A) Symbols: circle, 5% (vol/vol) ethanol; triangle, 10% (vol/vol) ethanol. (B) Symbols: circle, 1% (vol/vol) *n*-butanol; triangle, 2% (vol/vol) *n*-butanol.

3-4-3 Organic solvent tolerances of strain JA300 *acrR*IS *marR* and its ⊿*lon* mutant

In chapter 2, JA300 *acrR*IS *marR*, which had a nonsense mutation in *marR* and an insertion of IS5 in *acrR*, and this mutant displayed high-level organic solvent tolerance was constructed (42). *E. coli* strains in general cannot form colonies on an agar medium that is overlaid with organic solvents with $\log P_{ow}$ values less than 3.9. However, JA300 *acrR*IS *marR* can form colonies on an agar plate overlaid with a mixed solvent of cyclohexane ($\log P_{ow}$, 3.4) and *p*-xylene ($\log P_{ow}$, 3.1). In an attempt to further improve the organic solvent tolerance of JA300 *acrR*IS *marR*, we constructed JA300 *acrR*IS *marR* ⊿*lon* and compared its organic solvent tolerance with that of JA300 *acrR*IS *marR.* The colony-forming efficiencies of these strains were measured on the agar plate overlaid with pure *n*-hexane, pure cyclohexane, and a mixture of cyclohexane (Fig. 3-6). Contrary to our expectation, JA300 *acrR*IS *marR* ⊿*lon* was more susceptible to organic solvents than JA300 *acrR*IS *marR*, although the tolerance level in JA300 *acrR*IS *marR* ⊿*lon* was higher than that in JA300 and slightly higher than that in JA300⊿*lon*. Thus, it was found that accumulation of these mutations reduced the solvent tolerance level in *E. coli*.

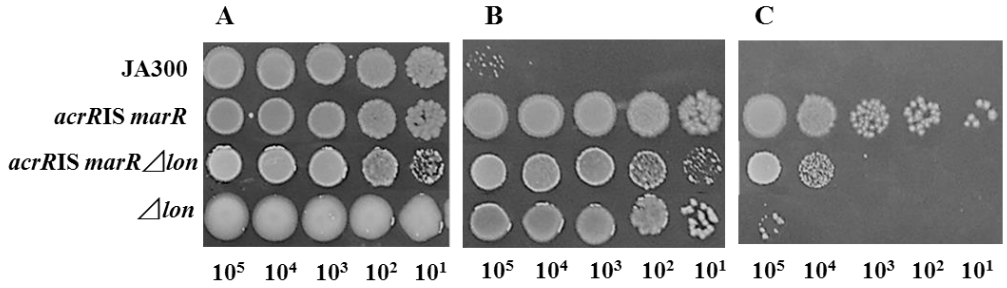

Fig. 3-6 Organic solvent tolerances of strain JA300 *acrR*IS *marR* and JA300 *acrR*IS *marR*⊿*lon* mutant

Colony-forming efficiency of JA300, JA300 *acrR*IS *marR* and JA300 *acrR*IS *marR* ⊿*lon* on LBGMg agar medium in the absence of an organic solvent (A) and in the presence of *n*-hexane (B), and cyclohexane (C).

3-4-4 AcrB levels in *E. coli* ⊿*lon* mutants

Mutations in *lon* can increase the expression level of the AcrAB-TolC efflux pump (20). The AcrB levels in ⊿*lon* mutants were investigated by immunoblotting analysis (Fig. 3-7). The AcrB levels in BW25113⊿*lon* and BW25113⊿*lon*⊿*wcaJ* were about twofold higher than that in the parent strain BW25113 (Fig. 3-7 A). The AcrB level in BW25113⊿*lon* was similar to that in BW25113⊿*lon*⊿*wcaJ*, indicating that disruption of the *wcaJ* gene did not affect the expression level of AcrB. JA300 *acrR*IS *marR* significantly increased the expression level of *AcrB* (Fig. 3-7 B). The *AcrB* level in JA300 *acrR*IS *marR* ⊿*lon* was about half of that in JA300 *acrR*IS *marR,* but was about twofold higher than that in strain JA300.

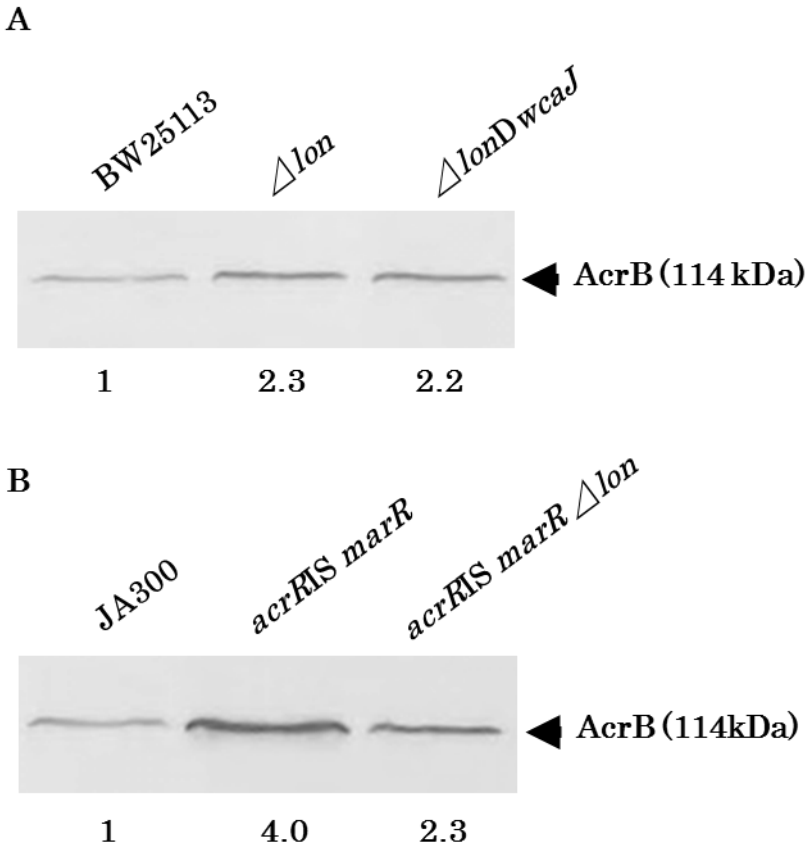

Fig. 3-7 Western blot analysis of AcrB

Western blot show the AcrB expression in BW25113 mutants (A) and JA300 mutants (B). Total cell lysate proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with polyclonal anti-AcrB antibodies. The expression ratio compared to the AcrB level of the parent strain is shown below each lane. (A) The strains were assigned to the following lanes: BW25113 (lane 1), BW25113⊿*lon* (lane 2), and BW25113⊿*lon*⊿*wcaJ* (lane 3). (B) The strains were assigned to the following lanes: JA300 (lane 1), JA300 *acrR*IS *marR* (lane 2), and JA300 *acrR*IS *marR* ⊿*lon* (lane 3).

3-4-5 Antibiotic susceptibilities of the ⊿*lon* mutants

Organic solvent tolerant *E. coli* mutants are known to exhibit resistance to a variety of antibiotics (5, 6). The antibiotic susceptibilities of strains BW25113, BW25113 ⊿ *lon* and BW25113 ⊿ *lon* ⊿ *wcaJ* to each of chloramphenicol, novobiocin, and ofloxacin were investigated by assessing the MICs (Table 3-3). It has previously been shown that *E. coli* strains overexpressing the AcrAB-TolC pump become resistant to these antibiotics (11). It was found that the MICs of strain BW25113 to these antibiotics were as follows: chloramphenicol, 6 μg/ml; novobiocin, 1600 μg/ml; ofloxacin, 0.4 μg/ml. On the other hand, the MICs of BW25113⊿*lon* and BW25113⊿*lon*⊿ *wcaJ* to these antibiotics were identical and as follows: chloramphenicol, 12 μg/ml; novobiocin, 1600 μg/ml; ofloxacin, 0.4 μg/ml. Although BW25113⊿*lon* and BW25113⊿*lon*⊿*wcaJ* exhibited higher chloramphenicol resistance than BW25113, the levels of resistance of these mutants to novobiocin and ofloxacin were similar to those of BW25113. It has been reported that *lon* mutants of *E. coli* did not produce a significant increase in multidrug resistance, despite their overproduction of the AcrAB-TolC efflux pump (20). These mechanisms are not *yet* fully understood. This study also showed that the ⊿ *lon* mutant did not exhibit a remarkable multidrug resistance-phenotype.

Table 3-3 Antibiotic tolerance of organic solvent-tolerant *E. coli* mutants

Strain	MIC (μg/ml) for:		
	CHL	NOV	OFL
BW25113	6	1600	0.4
BW25113△*lon*	12	1600	0.4
BW25113△*lon*△*wcaJ*	12	1600	0.4

Abbreviations: CHL, Chloramphenicol; NOV, Novobiocin; OFL, Ofloxacin.

3-5 Conclusion

The Lon ATP-dependent protease plays a major role in protein quality control and thus contributes to many biological processes in bacteria (37, 41). Although the Lon protease is involved in the regulation of numerous pathways, it is a nonessential enzyme under normal growth conditions in many bacteria (41). *E. coli lon* mutants become sensitive to DNA-damaging agents and accumulate abnormal proteins. In this study, it was showed that disruption of the *lon* gene was able to improve organic solvent tolerance in *E. coli*. Colonies of *E. coli lon* mutants present a mucoid phenotype, because the *lon* mutants overproduce the capsular polysaccharide due to the stabilization of the transcriptional activator RcsA (35). Extracellular polysaccharide has been reported to be involved in organic solvent tolerance in several bacteria. These correlations between capsule polysaccharide production and organic solvent tolerance are indicative of common survival strategies to withstand organic solvent stress. Therefore, it was assumed that the overproduction of capsule polysaccharide might improve organic solvent tolerance also in *E. coli*. However, this study showed that the organic solvent tolerance of nonmucoidal BW25113⊿*lon*⊿*wcaJ* was similar to that of BW25113⊿*lon*. This suggested that the overproduction of capsular polysaccharide is not involved in the improvement of organic solvent tolerance in *E. coli*. On the other hand, it has been reported that disruption of the *lon* gene can enhance the expression level of the AcrAB-TolC pump (20). This study also showed that the expression level of AcrB protein in BW25113⊿*lon* was higher than that in BW25113. To clarify the involvement of the AcrAB-TolC pump in

organic solvent tolerance in BW25113⊿*lon*, the organic solvent tolerance of BW25113⊿*lon*⊿*acrB* was examined. The results showed that BW25113⊿*lon*⊿*acrB* was highly sensitive to organic solvents. Thus the increase in the AcrAB-TolC efflux pump was the main cause of the improved organic solvent tolerance in the ⊿*lon* mutant. BW25113⊿*lon*⊿*acrB* became more sensitive to *n*-nonane than BW25113⊿*acrB*. Thus, disruption of the *lon* gene seemed to further decrease the solvent tolerance of a mutant lacking AcrAB-TolC pump activity. BW25113 ⊿ *lon* also exhibited higher organic solvent tolerance in the LBGMg liquid medium than the parent strain. However, the tolerance of BW25113⊿*lon* against hydrophilic solvents such as ethanol and *n*-butanol was not improved but partially lowered. Because hydrophilic solvents have higher affinity to hydrophilic capsular polysaccharide than hydrophobic solvents, the *lon* mutant might be sensitive to hydrophilic solvents rather than hydrophobic solvents. On the other hand, it has been reported that the AcrAB-TolC pump is not involved in the tolerance against short-chain alcohols (2).

The JA300 *acrR*IS *marR* overexpressing the AcrAB-TolC pump exhibited the highest organic solvent tolerance among about 100 cyclohexane-tolerant *E. coli* mutants (42). Mutations in *marR* can enhance the expression of MarA, and mutations in *lon* can increase the stability of MarA. Therefore, mutations both in *marR* and *lon* were assumed to significantly increase the expression level of the AcrAB-TolC pump and concomitantly enhance the organic solvent tolerance level in *E. coli*. In this study, we attempted to construct an *E. coli* mutant with high-level organic solvent tolerance by

disrupting the *lon* gene in JA300 *acrR*IS *marR*. However, the resulting strain JA300 *acrR*IS *marR* ⊿*lon* became more sensitive to organic solvents than JA300 *acrR*IS *marR*. In addition, the AcrB expression level in JA300 *acrR*IS *marR* ⊿*lon* was lower than that in JA300 *acrR*IS *marR*. These results suggested that accumulation of these three mutations seemed to decrease the level of AcrAB-TolC pump and thereby reduce the solvent tolerance level in *E. coli*.

It has been reported that *lon* mutants of *E. coli* did not produce a significant increase in multidrug resistance, despite their overproduction of the AcrAB-TolC efflux pump (20). T*hese* mechanisms are not yet fully understood. It was also found that the ⊿*lon* mutant did not show a remarkable multidrug resistance-phenotype. However, this study showed that *lon* disruption can significantly enhance the tolerance of *E. coli* against hydrophobic organic solvents, unlike in the case of multidrug resistance.

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Chapter 4

Conclusion and future perspective

Organic solvents are generally toxic to organisms including microorganisms. Organic solvents accumulate in and disrupt the cell membrane because they can bind to the cell membrane, thereby affecting its integrity. This results in the inhibition of microbial growth. Microbial cells respond, adapt and develop tolerance to organic solvents by multiple mechanisms. Various mechanisms related to organic solvent tolerance have been reported so far. In these studies, more is known about how cells respond to organic solvents. However, less is known about how to develop tolerant strains. This study focused on the development of organic solvent tolerant *E. coli.* In this final chapter, we are presenting the major observations of our research.

1. Cyclohexane tolerant mutant, strain CH7 was isolated from cyclohexane sensitive *E. coli* K-12 strain JA300. CH7 had mutations in regulatory genes both *marR* and *acrR*. The *marR* mutation in CH7 was a nonsense mutation leading to a translation termination codon at the position of E109 (marR109 mutation), and *acrR* was inserted by the insertion element IS5 (*acrR* IS mutatioin). To clarify the involvement of these mutations in organic solvent tolerance, the *marR* and/or *acrR* regions were introduced into the *E. coli* JA300 chromosome and JA300 *acrR*IS, JA300 *marR*, and JA300 *acrR*IS *marR* were constructed. Organic solvent tolerance of the constructed mutants was increased in the following

order: JA300 *acrR*IS < JA300 *marR* < JA300 *acrR*IS *marR*. JA300 *acrR*IS *marR* showed similar organic solvent tolerance level as strain CH7 in the presence of the solvents tested. In addition, the expression level of AcrAB-TolC efflux pump in JA300 *acrR*IS *marR* was improved compared to that of JA300, and was also similar to those in strain CH7. Furthermore, the intracellular amounts of organic solvents in JA300 *acrR*IS *marR* were kept lower than that in JA300, and the level was similar to those in CH7. JA300Δ*acrA* *acrR*IS *marR* became sensitive to organic solvents. In addition, the tolerance level of JA300Δ*acrA* *acrR*IS *marR* was similar to that of JA300Δ*acrA*. This result indicated that the increase of organic solvent tolerance in JA300 *acrR*IS *marR* was caused by overexpression of AcrAB-TolC efflux pump. Therefore, we clarified that only two mutations in regulatory genes in *acrR* and *marR* confer high-level organic solvent tolerance on *E. coli* (Fig. 4-1).

2. We found that mucoid phenotype strain BW25113⊿*lon* exhibited higher organic solvent tolerance than parent strain BW25113 in the presence of a mixture of *n*-hexane and cyclohexane. To clarify the involvement of the AcrAB-TolC efflux pump and capsular polysaccharide in organic solvent tolerance in BW25113⊿*lon*, organic solvent tolerances of BW25113⊿*lon* ⊿*acrB* (mucoid phenotype) and BW25113⊿*lon*⊿*wcaJ* (non-mucoid phenotype) together with BW25113⊿*acrB* and BW25113⊿*wcaJ* were examined. The organic solvent tolerances of BW25113⊿*wcaJ* and BW25113⊿*lon*⊿*wcaJ* were similar to those of the BW25113 and

BW25113 ⊿ *lon* strains, respectively. These results indicated that capsular polysaccharide is not involved in organic solvent tolerance. In addition, BW25113 ⊿ *lon* ⊿ *acrB* and BW25113 ⊿ *acrB* were highly sensitive to organic solvents. Thus, it was found that organic solvent tolerance in the ⊿*lon* mutant depends on the AcrAB-TolC pump (Fig. 4-1). In addition, this study showed that we can construct an organic solvent tolerant strain by introducing the *lon* mutation.

The use of two phase systems consisting of organic solvent and aqueous medium provides numerous attractive advantages in the bioconversion of various chemicals by whole-cell biocatalysts. The advantages of two-phase systems include not only the production of valuable compounds from hydrophobic raw materials, but also the maintenance of a low concentration of toxic or inhibitory compounds in the aqueous phase and an easier recovery of both product and biocatalyst. However, the catalytic efficiency of whole-cell biocatalysts in two phase systems is often hindered by the toxicity of organic solvents toward the cells. Thus, microorganisms with organic solvent tolerance can be efficient biocatalysts in two phase systems.

This study suggests a new strategy for increasing the organic solvent tolerance level in *E. coli* to improve the usability of the whole-cell biocatalysts in two phase systems employing organic solvents.

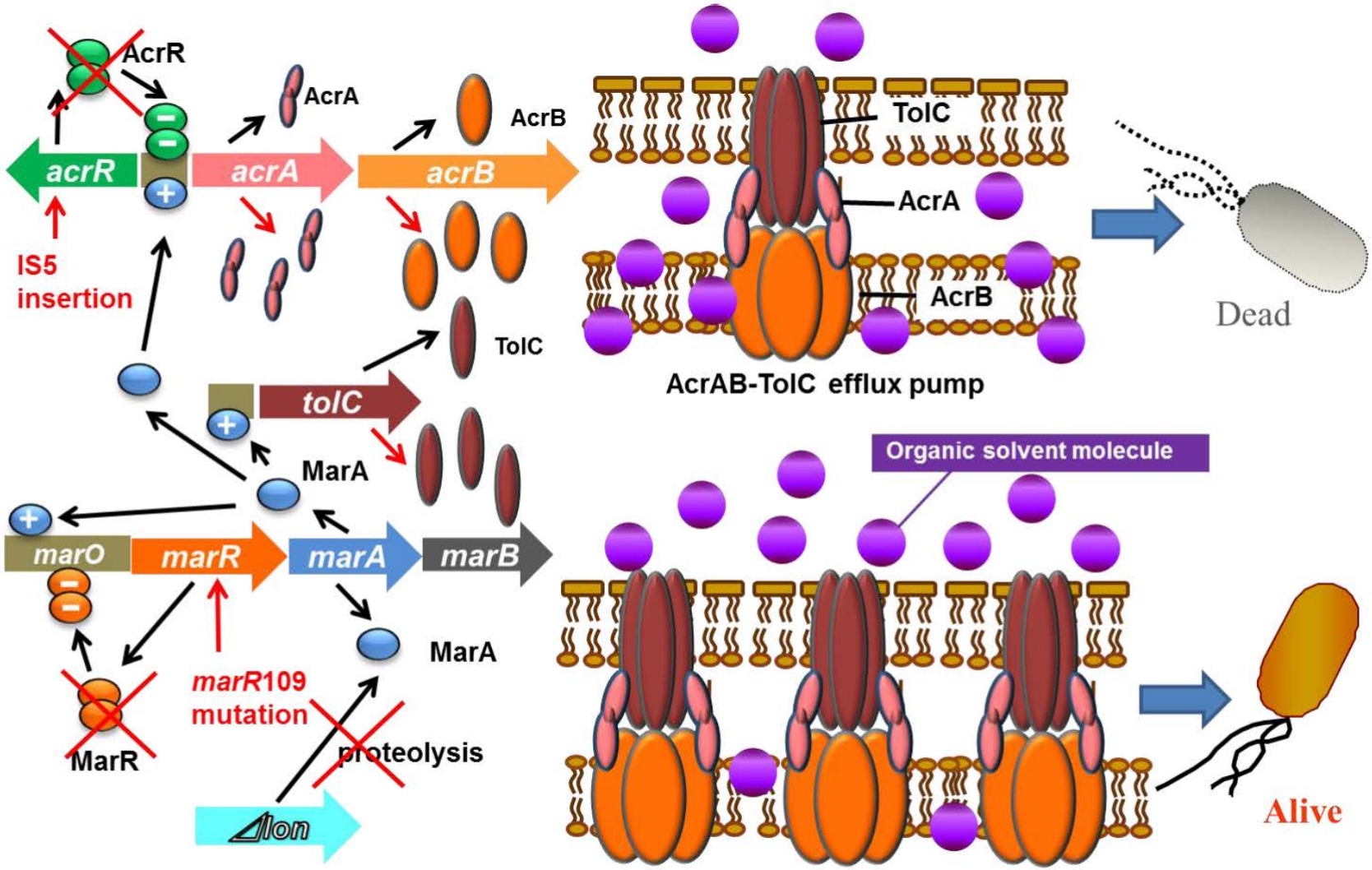

Fig. 4-1 Improvement of organic solvent tolerance by mutations in *acrR*, *marR*, and *lon* in *E. coli*
Gene mutations in a*crR*, *marR*, or *lon* can increase AcrAB-TolC pump. This leads to improve organic solvent tolerance in *E. coli* .

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